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# BACTERIOLOGICAL REVIEWS

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# THE ADAPTIVE PRODUCTION OF ENZYMES BY BACTERIA

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Wortmann was probably the first physiologist to describe in biochemical terms the mechanism by which living cells can adapt themselves to the utilization of certain foodstuffs (83). He observed that the cells of an unidentified bacterial species produced amylase when grown in media containing starch, whereas this enzyme failed to appear when the same culture was grown in media which did not contain this polysaccharide. In the same paper Wortmann also recognized that, on the contrary, yeast always produces invertase, whether or not sucrose is a constituent of the medium. Since then, the influence of the composition of the culture medium on the enzymatic activity of microbial cultures has been analyzed by many investigators and it became recognized that the production of a given enzyme might be greatly stimulated when the substrate which it attacks is a constituent of the culture medium. In the appended bibliography, an attempt has been made to present a fairly complete list of the publications dealing with this subject.

Karström designated as "adaptive" those enzymes which are produced as a specific response to the presence of the homologous substrate in the culture medium. He differentiated them from the "constitutive" enzymes which are always formed by the cells of a given species, irrespective of the composition of the medium (45, 48, 49).

Karström's terminology is convenient and has found wide acceptance. It is not sufficient, however, to describe fully the complex influence which the environmental factors, and in particular the composition of the culture medium, may exert on the

enzymatic constitution of the microbial cell. For instance, cultures of *Proteus vulgaris* grown in media containing *l*-leucine or *d*-isoleucine are very rich in urease; when grown in Uschinsky's synthetic medium, however, the same bacterial species forms only traces of urease although catalase is abundantly produced (43, 62). It is also known that calcium bears a definite relation to the formation of gelatinase by some bacterial species (34, 35, 56). In other words, the production of a microbial enzyme may be stimulated by the presence in the medium of substances unrelated to the substrate attacked by the enzyme (65, 66).

On the other hand, the stimulating effect of a given substrate upon the production of the homologous enzyme may exert itself through a number of different mechanisms. For instance, we may be dealing with a microbial culture capable of producing a variant endowed with a new enzymatic property. If the substrate attacked by this new enzyme is present in the medium, the variant may be favored to multiply rapidly, and the new enzyme will accumulate in the culture.

*Escherichia coli-mutabile* offers an example of this mechanism which has been carefully analyzed. This bacterial species usually does not produce the enzyme lactase; it becomes capable, however, of rapidly fermenting lactose after repeated transfers in lactose broth. Lewis has made the important observation that *Escherichia coli-mutabile* always produces a few cells (in a ratio of 1:1,000,000) capable of attacking lactose, even when grown in lactose-free media (53). It is obvious that the presence of lactose in the medium will favor the multiplication of these lactose-fermenting variants, and therefore stimulate the accumulation of lactase in the medium. Thus, many cases of "training" are undoubtedly due to a natural selection of a variant form. It is a characteristic of the new properties—a new enzymatic function, for instance—which are the results of natural selection, that they develop slowly and progressively in successive transfers of the culture in the specific medium which calls them forth. Once developed, however, the new property appears stable for a number of generations, even though the culture is transferred

repeatedly into media not containing the substrate with reference to which variation has taken place (36, 52, 55, 59, 62, 63, 64).

It is in this respect that the "adaptive" enzymes considered in the present review exhibit a distinctive, different behavior. They appear without delay when the cells of the proper microbial species start multiplying in a medium containing the specific substrate; the specific enzymatic activity reaches its maximum development during growth of the very first transfer into the specific medium, but the enzymes again fail to be formed as soon as the cultures are transferred to media lacking the specific substrates. An adaptive enzyme therefore appears as a specific response of the cell to the presence of a given substance in the medium; it does not conform to the behavior of hereditary variations.

#### MECHANISM OF FORMATION OF ADAPTIVE ENZYMES

It is well known that the enzymatic activity of the *intact* living cell is affected by a great many factors, such as the age of the culture, cellular permeability, etc. (2, 65, 66, 80, 81, 82). It is not possible, therefore, to determine quantitatively the concentration of a given enzyme present in a culture from the rate of the reaction observed when the living cells are used as source of enzyme. In other words, increase in enzymatic activity of a culture does not necessarily mean increased production of enzyme. In many instances, however, the presence of adaptive enzymes has been demonstrated by testing cells killed with toluol (46, 47), or filtered autolysates (9, 10, 12, 14) under conditions where factors of cellular permeability could not vitiate the interpretation of the results. It remains possible, however, that in other cases, an increase in enzymatic activity, or the appearance of a new enzymatic function may be due, not to the production of a new enzyme, but to other mechanisms which result in the activation of enzyme systems already present (61).

Moreover, it is likely that in many, if not all cases, the adaptive response may take the form of an increased production of the specific enzyme, rather than the formation of a new enzyme.

Euler, for instance, found that invertase is produced by *Escherichia coli* on many media not containing sucrose, but that the production of this enzyme is increased many times (40 times in some cases) when sucrose is a component of the medium (18, 19, 21 to 26).

As stated earlier in this review, adaptive enzymes are characterized by the fact that they appear and reach their maximum activity during growth of the first transfer of the culture in the specific medium, and fail to be formed as soon as the culture is again transferred into a medium not containing the specific substrate. It appears unlikely that a character acquired and lost so suddenly could be due to natural selection of variant forms endowed with the new enzymatic property. Against the natural selection hypothesis is also the fact that in many cases the newly acquired enzyme can hardly be of any value to the organism which produces it. Yudkin, for instance, offers as an illustration the adaptive production of hydrogenlyase by *Escherichia coli* grown in a formate medium (84, 85, 86). The reaction  $\text{HCOOH} = \text{H}_2 + \text{CO}_2$  which is catalysed by hydrogenlyase can at best liberate only very small amounts of free energy, and it is unlikely that the products of the reaction are required by the organism for its growth. He remarks also that, on the basis of the natural selection hypothesis, the immediate loss of the hydrogenlyase when the organism is transferred to a medium deficient in formic acid would suggest that the loss of the enzyme is an advantage to the cells grown in plain broth, an unlikely assumption. From these considerations, Yudkin decides that the production of hydrogenlyase can not be due to natural selection.

The conversion of creatine into creatinine by an adaptive enzyme is another example of a reaction which appears to be of little use to the organism involved (14). Even more striking, however, is the fact that the enzyme is formed equally well when creatinine (the end product of the reaction) instead of creatine is added to the medium. This observation is of some theoretical significance.

It is obvious that the natural selection hypothesis would be ruled out if the production of adaptive enzymes could be obtained

in the absence of any cellular division. Stephenson and Stickland working with hydrogenlyase (71, 72, 73) and Stephenson and Yudkin (74) working with yeast galactozymase, claim to have established this fact. Their conclusion is based on the following evidence: a) washed cells not possessing the enzyme were re-suspended in a solution of the specific substrate, and enzyme formation could be demonstrated within one hour, *i.e.*, in a time too short for appreciable cell division to take place; b) on the basis of viable and total cell counts during the adaptation, it was found that enzyme production occurred without increase in cell numbers (the results were considered accurate within 5 per cent).

These observations suggest strongly that enzyme production can occur in the absence of cell multiplication; they do not, however, mean that enzyme production can occur without the synthesis of new protoplasm. It is a fact that most bacterial cells, when transferred to a new medium, undergo a phase of enlargement and elongation prior to cell division (40, 41, 68, 79). During this period, the metabolism of each individual cell increases, a phenomenon probably associated with the production of new protoplasm not accompanied by cell division. It must be mentioned at this point that, in spite of many attempts by several investigators, it has been found impossible to observe the production of enzymes in the presence of protoplasmic poisons or under conditions incompatible with cell growth (9, 10, 20, 74). Moreover, even in the case of formic hydrogenlyase which was produced in the absence of cellular multiplication, Stephenson and Stickland (72, 73) observed that no enzyme was formed unless some bouillon was added to the formate solution. It seems, therefore, that although the adaptive production of enzymes can occur in the absence of cellular division, it always involves the synthesis of new protoplasm. Hegarty has shown that physiologically young cells adapt themselves much more rapidly than older cells (38).

Some authors have tried to describe the mechanism of enzyme production in chemical terms. Quastel (65, 66) showed that, although the production of catalase, urease and fumarase by



*Micrococcus lysodeikticus* varies greatly according to the medium in which the organism is grown, the presence of urea does not stimulate urease, nor does that of succinate or fumarate stimulate fumarase production. It will be recalled also that the production of certain enzymes (urease and gelatinase) is conditioned by the presence in the culture medium of substances entirely unrelated to the homologous substrate (34, 35, 43, 56, 62, 65, 66). All these results, according to Quastel, are best interpreted by assuming that the enzymes are themselves metabolites, whose rate of formation and destruction varies with the conditions of growth. The effect of the substrate could then be due either to contributing the necessary organic molecules for the synthesis of the enzyme, or to affecting its stability (for instance by combining with it). The adaptive stimulation caused by the homologous substrate would only be one particular application of these principles.

Yudkin (86) formulated a "mass action theory" of enzyme production in which he assumed that, in all cases, the stimulation caused by the proper substrate does not result in the production of a new enzyme, but only increases the production of an enzyme otherwise formed in small amounts. He postulated also that in the cell the enzyme is in equilibrium with a precursor. Any substance combining with the active enzyme would then disturb the equilibrium and thus cause the production of more enzyme from the precursor; the adaptive stimulation by the homologous substrate could be explained on this ground. Yudkin has offered many facts in support of his theory; there are a few, however, which are perhaps in conflict with it. Both in the case of the organism which hydrolyses the capsular polysaccharide of Type III pneumococcus, and of the organism which decomposes creatinine, the addition of 1 to 2 per cent casein hydrolysate to media containing the specific substrates retards markedly the appearance of the homologous enzyme, although it does activate and increase growth. The final yield of enzyme is not decreased; only its rate of production is slower. It has been observed that the casein hydrolysate is more readily utilized than either the polysaccharide or creatinine and that its presence in the medium

retards the decomposition of the two latter substances. It would seem, therefore, that the formation of the specific enzyme depends not upon the presence of the homologous substrate in the medium, but upon its utilization by the metabolizing cell.

Wooldridge (82) pictures the cell surface as "comparatively loosely-knit structures composed largely of complex organic molecules, the latter orienting themselves in the surface as the result of the affinities exhibited between the various groupings of that molecule and those possessed by the adjacent molecules in the surface and the molecules in true solution on either side of the surface." In this way, a substrate present during the growth of a cell will tend to increase the concentration of enzymes available to act on that substrate. In some respects this hypothesis recalls the mechanism invoked by Breinl and Haurowitz (4) and by Mudd (58) to explain how an antigen determines the specific configuration of an antibody molecule (67); these authors suggested that during the synthesis of antibody globulin, the arrangement of amino acids is modified by the polar forces of the antigen in contact with the structure where the antibody is produced. It is worth repeating here that the formation of adaptive enzymes probably takes place at a time when new protoplasm is synthesized by the bacterial cell.

#### THE SPECIFICITY OF ADAPTIVE ENZYMES

Adaptive enzymes exhibit a remarkable specificity toward the substrates which have stimulated their formation. Karström has shown, for instance, that the adaptive enzymes of *Betacoccus arabinosaceus* can differentiate between different monosaccharides and polysaccharides (45, 48). Diehl has claimed that casein and gelatin can be differentiated by the adaptive enzymes of some proteolytic organisms, but his results need to be confirmed by more accurate methods (7, 52).

The enzymes which hydrolyze the capsular polysaccharides of pneumococci differentiate between polysaccharides which give rise to cross reactions in specific antisera; for instance, the polysaccharide of gum acacia which reacts in Type III pneumococcus antiserum, is not affected by the enzyme which hydrolyses the

Type III polysaccharide (12). Even more striking is the difference between enzymes attacking the polysaccharides of Type III and Type VIII pneumococcus (30, 39). Both of these substances are composed of glucose and glucuronic acid, in the ratio of 1:1 for Type III and 1:3 for Type VIII and exhibit cross reaction in immune sera. However, the bacterial enzymes developed against each one of the polysaccharides fail to attack the other; in other words, the enzymes are even more specific than the antibodies obtained by immunization of experimental animals with the capsular antigens of pneumococci (11, 69).

The specificity of the adaptive creatinine oxidases obtained from two bacterial cultures has been established by testing these enzymes against a number of substrates related to creatinine (13). It has been found for instance that the mere addition of a methyl or acetyl group to the creatinine molecule completely inhibits the enzymes; the removal of the methyl group in position 3 (leaving glycoyamidine), or its shift from position 3 to position 5 retards considerably the action of one of the bacterial enzymes and inhibits completely the other. The enzyme which converts creatine into creatinine exhibits a similar specificity (14).

#### APPLICATIONS OF MICROBIAL ADAPTIVE ENZYMES

Because of their specificity, adaptive enzymes have already found a place in the analysis of several biological and biochemical problems. Karström for instance observed that *Escherichia coli* adapts itself to the fermentation of maltose and lactose through the formation of the disaccharidases maltase and lactase (47); it is therefore likely that the direct fermentation of the disaccharides without preliminary hydrolysis which has been postulated by Willstätter in the case of certain yeasts, does not take place in bacterial processes. By using a strain of *Escherichia coli* which produces maltase, but not invertase, Karström (46) also established that, contrary to Weidenhagen's claim, maltose and sucrose are not hydrolyzed by the same alpha glucosidase.

F. H. Johnson (44), working with luminescent bacteria, observed a complete inhibition of respiration and luminescence by alpha-methylglucoside. This inhibition, however, is followed by

a sudden "escape" with complete recovery of both respiration and luminescence, which Johnson attributed to the production of an adaptive enzyme.

The mechanism of production of molecular hydrogen by *Escherichia coli*, and the possible intermediate production of formic acid, have been investigated by means of formic hydrogenlyase (61, 72, 73).

In the course of studies on renal function, it became necessary to develop a method for the quantitative estimation of the very small amounts of creatinine present in blood. The identification and analysis of creatinine in biological fluids have thus far depended on colorimetric methods which are so nonspecific, that many authors have denied the very presence of creatinine in circulating blood. With the help of specific adaptive enzymes, it has been demonstrated that creatinine is indeed present in blood plasma and in the erythrocytes. Quantitative studies of the amount of creatinine present in the blood and urine of normal and nephritic humans are being used in an analysis of renal function in health and disease (57).

The recently discovered adaptive anhydrase which converts creatine into creatinine, offers an interesting example of the enzymatic combination of an amino and a carboxyl group to form the CO—NH linkage; this reaction may be useful in the study of the metabolism of creatine (14).

A few years ago, an adaptive enzyme capable of hydrolysing the capsular polysaccharide of Type III pneumococcus was extracted in solution from the cells of a saprophytic bacterial species. Experiments with the enzyme afforded additional and final evidence as to the rôle played by the capsular polysaccharides in determining the serological specificity of pneumococci (12). It was also possible to protect experimental animals (mice, rabbits and monkeys) against large numbers of infective doses of Type III pneumococcus by injection of the soluble enzyme; this treatment, however, was entirely ineffective against pneumococci of other types (1, 29, 31). These results emphasize once more the rôle of the capsular polysaccharides in conditioning the virulence of encapsulated pneumococci (11).

All organic matter in nature eventually becomes the prey of microorganisms which break it down through the agency of their cellular enzymes; in fact there are usually found several species of microorganisms capable of attacking one same chemical entity and the enzymes through which different microbial species attack the same substrate vary in their mode of action. It is obvious therefore that one can find in the microbial world enzymes capable of performing almost every conceivable type of biochemical reaction, many of which are not known to take place elsewhere in the animal or plant kingdoms. In many cases, as we have seen, microorganisms adapt themselves to the performance of a given biochemical reaction by the production of specific enzymes. On account of their cellular origin these adaptive enzymes are capable of functioning under moderate chemical conditions (pH, temperature, etc.) and this property, together with their specificity render them ideal reagents for the analysis of biological problems. It is apparent, therefore, that the readiness with which microorganisms produce adaptive enzymes suggests a method which will yield an infinite number of specific "physiological" reagents.

It is also true, on the other hand, that the production of adaptive enzymes is a striking example, fairly well defined in biochemical terms, of adaptive response of the living cell to changes in the environment. A consideration of this phenomenon brings the bacteriologist back into the main channels of biological thought, to the biological problem *par excellence*, the problem of adaptation. The study of the mechanism whereby microorganisms produce those enzymes which appear as an adaptive response to the presence of the homologous substrates in the culture medium, bids fair to throw light on some of the reactions involved in biological adaptation.

#### SUMMARY AND CONCLUSIONS

The production of enzymes by microorganisms is influenced by different factors. Some bacterial species, for instance, give rise to variants endowed with new enzymatic properties; these are hereditary characters. Certain substances increase the yield

of a given enzyme by contributing the necessary organic or inorganic molecules for its synthesis, or by preventing its inactivation.

In other cases, the production of a given enzyme is greatly stimulated when the substrate which it attacks is a constituent of the culture medium. These "adaptive" enzymes appear and reach their maximum development during the growth of the first transfer of the culture in the specific medium; they fail to be formed as soon as the culture is again transferred to a medium not containing the specific substrate. Although the production of adaptive enzymes need not be associated with cellular multiplication, all evidence available indicates that it involves the synthesis of new protoplasm. It is suggested that the synthetic process is, so to speak, oriented or guided by the chemical structure of the substrate which thus determines the specificity of the enzyme.

Adaptive enzymes do in fact exhibit a remarkable specificity toward the substrates which have stimulated their production and they bid fair, therefore, to serve as useful tools in the analysis of many biological and biochemical problems.

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# THE MICROBIOLOGY OF INSECTS

## WITH SPECIAL REFERENCE TO THE BIOLOGIC RELATIONSHIPS BETWEEN BACTERIA AND INSECTS

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It is noteworthy that one of the first instances of an animal disease shown to be caused by a microorganism was Pasteur's discovery of the etiologic agent of pébrine, a microsporidian disease of silkworms. This dramatic beginning in the study of the relationships between microorganisms and insects does not, however, seem to have maintained itself. The only type of microbiologic-entomologic relationship that has been developed to any great extent is that of the rôle of arthropods as hosts and vectors of the agents of human, animal and plant diseases. Important as this aspect of the subject is, it should not be developed to the exclusion of other important relationships between microorganisms and insects. More intimate associations between the members of these two biologic groups are, with few excep-

tions, little known and appreciated. The problems arising out of these associations are relatively unexplored. Such relationships are of still greater consequence when there exists the possibility of transferring experimental results to the study of arthropod-transmitted diseases of man, animals and plants. Such possibilities have already been demonstrated in the case of the diseases due to viruses, rickettsiae, and other agents. Further investigation of the little-studied symbiotic associations between microbes and insects would greatly increase the knowledge of physiology not only of insects and microbes themselves, but of living things in general. The successful use of microbial agents as a method of insect control likewise rests largely upon a more thorough understanding of the intimate relationships between microorganisms and insects. These and many other important problems could be clarified and better understood if more knowledge existed concerning the microbiology of insects.

This review is not intended to be a compilation of the results of all the investigations made in this field. Instead, it is an attempt to systematize the subject and to assist in securing for it a place in the attention of those interested in microbiology and entomology. It should be emphasized that many of the biologic relationships between microorganisms and insects have, of necessity, not been considered in this review. In general, the discussion has been limited to what may be considered as the flora of normal insects.

If, as has often been said, the principal value of facts is that they give us something to think about, then a gathering and orientation of the main facts of the subject under discussion should be worthy of presentation. To a great extent it will be left to the reader to formulate his own opinions concerning much of the material presented. This necessity is demanded by the lack of sufficient data upon which to form definite conclusions. Nevertheless, certain generalizations are necessary at this stage if there is to be any further progress in the field. This the writer will attempt to do whenever the opportunity presents itself. It is fairly certain that many of the generalizations to be

made, as well as many of those made by other writers, will have to be modified and re-expressed as the field develops.

#### THE EXTRACELLULAR FLORA OF INSECTS

By reason of the ubiquity of microorganisms in nature it is not surprising that the intestinal tract of many insects should be found to contain large numbers of microbes. If one makes a microscopic examination of the contents of the alimentary canal of an insect, he may find all or any one of the chief microbial forms of life: bacteria, yeasts, molds, and protozoa. In most cases the bacteria predominate in numbers over the other forms. Moreover, one or several species of bacteria may be constantly represented in the flora of the digestive tract of any given species of insect.

##### *Flora of the cecal pouches*

Probably the first observer to record the presence of living microorganisms in insects was Raimbert (1869) who obtained anthrax bacilli from flies experimentally exposed to the cadavers of infected animals. Another of the early investigators to observe the presence of bacteria in the intestinal tract of insects was Forbes (1882). On examining crushed specimens of the chinch bug reared in the laboratory, he found the fluids to be "swarming with a species of *Bacterium* not easily distinguishable from *B. termo*."<sup>1</sup> This bacterium was found to be much more numerous in that part of the intestinal canal posterior to the malpighian tubes. Similar results were obtained from observations on chinch bugs taken from the field, although nothing of the sort could be detected in the fluids of the corn plant louse (*Aphis maidis*) which fed on the same stalks.

Later on, Forbes (1892) reported on the bacteria normal to the digestive organs of Hemiptera. He noted that certain appendages to the alimentary canal in the members of the families Scutelleridae, Pentatomidae, and in certain Lygaeidae and Coreidae contained large numbers of bacteria. In the Coreidae

<sup>1</sup> Unless otherwise indicated, all scientific names used in this review, regardless of how they are designated in present-day nomenclature, are those used in the publication cited.

and Lygaeidae these cecal structures were present in one genus and absent in another of the same family. Only the higher Hemiptera (Pentatomidae, Scutelleridae, Corimelaenidae, etc.) invariably possess them. They are always absent in the lower Hemiptera. In the absence of these structures, the microorganisms are not found in any other part. According to Forbes, the gastric pouches of grasshoppers, cockroaches, and carabid beetles do not commonly contain bacteria. In the Hemiptera the gastric ceca are located near the posterior end of the mid-intestine, while in many other insects, particularly the Orthoptera, Coleoptera, and Diptera, the ceca occur near the anterior end. Incidentally, in the light of a relationship to be discussed later, it is interesting to note that Forbes mentioned the fact that the fat bodies of various cockroaches contained structures that appeared to be bacteria. With respect to the chinch bug, this investigator stated that the organism most commonly present in the intestinal tract was one he called *Micrococcus insectorum*.

Continuing these studies, Glasgow (1914) described in greater detail the bacteria-containing ceca of the Heteroptera (Hemiptera) and revealed many interesting facts. The ceca of a given species of insect, regardless of the geographic source of the specimen, are invariably filled with a pure culture of a single species of bacterium. Morphologically, the bacteria from different hosts vary greatly, ranging from minute, coccus-like bacilli to huge spirochete-like forms; but in whatever insect they occur, they are morphologically characteristic for the particular species harboring them. Glasgow believed that these strikingly different forms of bacteria really belong to a single, clearly defined group and that the differences in structure are due to the effect of some specific physiologic peculiarity of the insect. It would be interesting to know what credence modern students of bacterial dissociation and variation would give this interpretation. (See also Paillot, 1931, 1932.) That these organisms are bacteria was indicated by culture experiments and agglutination tests. Most of the bacteria from the various species of insects could not be grown by Glasgow on ordinary culture media, although those from *Anasa tristis* were cultured in nutrient broth.

These bacteria, normally present, appear not only to inhibit the development of foreign bacteria, but to exclude them altogether. The mid-intestine is usually wholly free of the invading bacteria and protozoa commonly present in many related insects. According to Glasgow, this is probably the chief function performed by the cecal bacteria in the life processes of the host. Since the ceca of the Hemiptera appear to contain definite species of bacteria, the phylogenetic significance and function of the ceca are of interest. There are complete gradations from extremely simple to very complex forms. Glasgow concluded that one function of these appendages is merely to provide a safe place for the multiplication of normal bacteria. It would seem, however, that their presence is probably more intimately concerned with the life processes of the insect than merely that of serving teleologically as a haven for bacteria.

One of the most interesting features of this bacterium-insect association is that it is congenital, the organisms appearing early in the alimentary canal of the developing embryo. This fact also indicates that a close relationship with the insect exists, although convincing evidence of true symbiosis is lacking. Much work must still be done to determine the true rôle that the bacteria play in the life of the insect. Kuskop (1924) also deals with this in a paper containing a tabular survey of these bacteria-containing ceca.

#### *Flora of the digestive tract proper*

Although the novelty of the cecal bacteria induces one to give them first attention, the microbial flora of the digestive tract itself is no less important and interesting. It was realized early by those studying diseases of insects that before conclusions could be made concerning the abnormal flora of the insect, the normal flora would have to be studied. This was exemplified by the work of White (1906) in his studies on the diseases of bees. In this investigation he also studied the bacteria of the normal combs, pollen, honey, larvae, adults, and the intestines of healthy bees. Both on the surfaces of the adult bees and on the combs there occurred quite constantly a species of bacterium referred to as "Bacillus A," which White believed to be the organism

confused by some workers with *Bacillus alvei*, the cause of European foul brood. In the pollen and in the intestines of the adult bees a species referred to as "Bacillus B" occurred very constantly. From the combs, *Bacterium cyaneus*, *Saccharomyces roseus*, and a micrococcus were isolated and studied. The normal larvae were, as a rule, sterile, as was the honey from a healthy hive. It is interesting to note that White quite constantly found an anaerobe in the intestine of the healthy bee. In addition, he isolated from the intestine the following microorganisms: *Bacillus cloacae*, *B. coli communis*, *B. cholerae-suis*, *B. subgastricus*, *Bacterium mycoides*, *Pseudomonas fluorescens liquefaciens*, and two forms referred to as "Bacillus E" and "Saccharomyces F."

During the years following 1906, the bacterial flora of the housefly (*Musca domestica* L.) as well as that of several species of cockroaches aroused considerable interest (Longfellow, 1913; Barber, 1914). Jackson (1907) found as many as 100,000 human fecal bacteria in a single fly, and recognized that these bacteria might easily survive passage through the intestinal canal of the insect. Graham-Smith (1909) examined 148 flies caught in various parts of London and Cambridge. Of these, 35 (24%) possessed externally or internally, or both, bacilli belonging to the colon group. Later (1913), he reported that *B. prodigiosus* could be cultivated from the contents of the crop and intestine of the housefly in large numbers up to 4 or 5 days after inoculation, and was found surviving in the intestine up to 18 days. Graham-Smith also states that although it seems to have been proved that the spores of *B. anthracis* may survive after being ingested by fly larvae, most observers agree that such non-spore-bearing pathogenic organisms as *B. typhosus*, *B. enteritidis*, and *B. dysenteriae* derived from cultures and added to the food of the larvae are not present in the flies which emerge, except under very special and highly artificial conditions. Somewhat earlier, however, Bacot (1911) reported that when the food of some newly hatched larvae of *Musca domestica* was inoculated with a culture of *Bacillus pyocyaneus*, viable bacteria remained in the gut during metamorphosis.

Torrey (1912) found that flies examined up to the latter part

of June were free from fecal bacteria of human origin and carried a homogeneous flora of coccal forms. During July and August, periods occurred during which the flies examined possessed several millions of bacteria, alternating with periods in which the number of bacteria was reduced to hundreds. Bacteria of the colon type were first encountered in abundance during the early part of July. The bacteria in the intestines of the fly were 8.6 times as numerous as those occurring on the external surface of the insect. Another example of seasonal incidence has been observed in the case of the bacteria producing soft rot of potatoes. In this case, the bacteria pass the winter in the digestive tract of the puparia of *Hylemyia cilicrura* Rond. (Leach, 1933). Nicoll (1911), and Cox, Lewis, and Glynn (1912) also studied the numbers and varieties of bacteria associated with the housefly, finding large numbers of the coliform type. (See also Hewitt, 1914.)

Bacot (1914) made similar studies on the bacteria of the alimentary canal of fleas during their metamorphosis, and found that the alimentary canal of the flea larva may become "infected" with the following bacteria if they are mixed with its food: *B. pyocyaneus*, *B. enteritidis*, *Staphylococcus aureus*, and *S. albus*. He also showed that an infection of the larval gut may persist until the resting period of the larva in the cocoon, and that there is no satisfactory evidence that such an infection can survive the pupal stage.

From this example, it can be seen that a knowledge of the fate of the microbial flora of an insect during metamorphosis is important from a practical standpoint; yet almost nothing is known about this matter. Bacot (1911) recognized the importance of the persistence of a bacterial "infection" in the larval gut of an insect through the period of metamorphosis and its survival in the adult after emergence from the pupa. This fact is especially important from a public health standpoint. If, for example, the larva of the housefly becomes a reservoir for organisms pathogenic to man and then transmits them when it is an adult, the chances of spreading disease are much greater. Furthermore, in deciding what is the normal flora of an adult insect,



one must consider adventitious organisms that have been picked up not only by the imago itself, but by the larva as well.

The bacterial flora may vary in different parts of the alimentary tract. For example, the milkweed bug, *Oncopeltus fasciatus* Say, has a distinctly different bacterial flora in its pylorum and rectum from that in the four stomachs which precede them (Steinhaus, 1940). The predominant bacterium isolated from the pylorum and rectum is one closely associated with the *Proteus* group of bacteria, while in the four stomachs the main flora consists of a species of the genus *Proteus* which differs from that found in the pylorum and rectum, and an organism very similar to those of the genus *Eberthella*.

*Numbers of organisms with respect to location in insect*

The numbers of organisms may also vary with the region in which they are located. The digestive tracts of some insects have been found to be sterile while others are packed with organisms. Hertig (1923) points out that in the honey bee the greater number of organisms is found in the hind intestine, particularly in the rectum, while relatively few both in numbers and variety are found in the ventriculus, except at times of food accumulation. In fact, this worker states that at times he obtained no growth at all in the media inoculated with a small section of the wall and contents of the ventriculus. He explains that this slight bacterial content of the ventriculus is due, perhaps, to the fact that solid particles pass rapidly to the hind-intestine, and further, that the contents of the ventriculus are at times rather acid, which may inhibit the multiplication of organisms. Stammer (1929) in a study of 37 species of trypetids showed the presence of bacteria in all cases. Their manner of distribution varied with the genus of the host. In the simplest case the bacteria were diffuse or in clumps in the intestinal contents of the larva and young adults. In old adults they were always present in enormous numbers in the lumen of the intestine. In the larvae of Tephritini, Schistopterini, and *Dacus oleae*, the diverticulum of the esophagus contains the bacteria. Melampy and MacLeod (1938) state that in the case of *Agriotes mancus* Say, the greatest number of bacteria was

found in the hind-gut. Thorpe (1930) reported a similar condition in the petroleum fly (*Psilopa petrolii*).

It is definitely known that the digestive tract of some insects, such as certain members of the blood-sucking group, is sterile. In some cases only certain parts of the tract are devoid of microorganisms. An example of this regional sterility is represented by blow-fly maggots used in the treatment of slow-healing wounds such as in osteomyelitis. Using the larvae of the blow-fly *Lucilia sericata*, Robinson and Norwood (1933, 1934) found that large numbers of the bacteria taken in with the food were destroyed in passing through the long, tubular stomach of the maggot. No viable bacteria were found in any cultures of the intestine. However, in all of the specimens dissected, abundant bacterial growth was obtained from the fore-stomach. The intermediate area, the hind-stomach, showed slight growth of microorganisms in one-third of the cases. (See also Simmons, 1935.) Duncan (1926) found that the feces of certain other insects were sterile. This worker also studied the nature of the bactericidal properties of insect feces. Nuttall (Herms, 1939) found that the anthrax bacillus died in the stomach of the bed bug in 48 to 96 hours at 13° to 17°C. and in 24 to 28 hours at 37°C., although the feces from the bugs contained living bacilli during the first 24 hours after feeding. Chapman (1924) examined the digestive tract of the confused flour beetle, *Tribolium confusum* Duv., and failed to find any living organisms present.

#### *The nature and kinds of bacterial flora*

As to the nature and kinds of bacteria comprising the flora of insects, not very much evidence exists. Very few investigators have attempted to identify or classify properly the bacteria they have isolated. For this reason we frequently see an organism referred to simply as a bacillus, coccus, bacterium, or cocco-bacillus. Because of these indefinite and ambiguous terms the true nature of the bacterial flora is not clearly defined. Just what types and groups of bacteria predominate in insects is difficult to say with certainty. A recent survey of the bacterial flora of certain insects (Steinhaus, 1940) indicates that most of the major

types of bacteria are represented. These include gram positive and negative short rods, gram positive spore-forming bacilli, and gram positive cocci. The gram negative short rods predominate, comprising slightly more than 50 per cent of the bacterial flora of the intestinal tracts of the insects studied.

Among other things, the reviewer's survey revealed a number of bacteria which were elliptical in shape and which one is prompted to designate as coccobacilli. They varied in their physiologic characteristics as well as in their reaction to the gram stain. These forms, however, may be pleomorphic types of the familiar short rods and cocci. The writer is not willing to go as far as have some workers, such as Pospelov (1926), who states that each species of insect has its own species of coccobacillus. Certainly, experimental evidence does not warrant such a sweeping statement. Nevertheless, it does appear that the presence of the coccobacillus, as a morphologic type, is characteristic of insects and this form may merit a taxonomic grouping of its own after a more thorough investigation of the subject. It is evident that steps should be taken to make clear just what is meant by the term "coccobacillus." Glaser (1918), in undertaking a systematic study of a number of cultures which were designated as *Coccobacillus acridiorum* d'Herelle, found that some of the separate cultures proved to be either different species or varieties of the same species. "This fact," says Glaser, "may account for some of the contradictory views held by so many workers and it is my hope that this article will also demonstrate the need for attention to the ordinary principles of bacteriology which seem to be so persistently neglected by many entomologists." (See Paillot, 1913; DuPorte and Vanderleck, 1917.)

Many peculiar forms are found in the bacterial flora of insects. During an investigation by Roberts (1935) of the intestinal flora of several termites from central Texas, a peculiar bacterial species was observed as a normal inhabitant of the termite intestine. The outstanding characteristic of the organism, which he named *Bacillus rotans*, is the mobility of young colonies on the surface of nutrient agar. (See also Smith and Clark, 1938; Clark, 1939.)

*Non-bacterial flora of insects*

Besides bacteria, other microorganisms are frequently found associated with insects. Rickettsiae, yeasts, molds, as well as protozoa have all been found living freely in the intestinal tract of arthropods. Jungmann (1918) noticed that *Rickettsia melophagi* was present in the stomach lumen and on the surface of the stomach epithelium of mature individuals of the sheep louse, *Melophagus ovinus*, but only rarely in the young individuals. The higher fungi, particularly the molds, have frequently been isolated from insects. Schaudinn (1904) was perhaps the first to describe a fungus as a significant normal inhabitant of an insect. In the mid-intestine of several species of Culicidae he found a fungus which he believed produced an enzyme that passed into the wound during the act of sucking and not only prevented coagulation of blood but also caused subsequent irritation and swelling.

With insects, as with other animals, the main association of viruses has been in connection with insect, plant and human diseases. Perhaps with the increase of our knowledge of viruses themselves and the perfection of techniques in the study and detection of non-pathogenic viruses, it will be found that insects harbor even these agents as normal inhabitants. It is noteworthy that insects have been found to harbor bacteriophage (Glaser 1938).

## THE RÔLE OF MICROÖRGANISMS IN THE NUTRITION OF INSECTS

Since microorganisms maintain such an intimate relationship with insects and since such a large number is harbored in the intestinal tract, one of the first probable effects of this relationship is that of the influence of microorganisms on insect physiology and nutrition.

*Bacteria as a source of food*

Bacteria may not only be related to the food habits of an insect, but they may also serve as food itself. The literature on this phase of the subject is both scattered and contradictory. It will be our purpose here merely to indicate the nature of the work

done and thus to reveal the fertility of the field for further research.

Mitchell (1907) early expressed the belief that the "wiggler" of *Stegomyia fasciata* is preëminently a bacteria-feeder, because the larvae develop rapidly in water contaminated with sewage. In later years her belief was supported by the work of Bacot (1916), Atkin and Bacot (1917), Barber (1928), Rozeboom (1935) and others. That mosquito larvae may live in the absence of bacteria has been shown by Trager (1935a, 1935b). In Bacot's report the suggestion that the bacteria themselves served as food for the mosquito larvae was based on the clearing action the latter displayed in water, originally turbid from its enormous bacterial content, in conjunction with the fact that the gut-contents of larvae taken from this water showed relatively few bacteria. He attributed the scarcity of bacteria to their being rapidly digested. Barber found that a combination of bacteria with infusoria or with algae seemed to afford the best conditions for the growth of *Culex quinquefasciatus* and of *Aedes aegypti*. No considerable growth of larvae was obtained in sterile nutrient media nor in cultures of the insect provided only with dead organic material. Howland (1930) observed that algae were ingested by many species of mosquito larvae and appeared to form an important part of their food. (See also Senior-White, 1928; Hinman, 1933.) A relationship similar to that of the mosquito larvae in contaminated water was suggested by von Wolzogen Kühr (1932) with the larvae of *Chironomus plumosus* which frequented sand-filters in the summer. This was attributed to the presence in the filters of *Pseudomonas fermentans* upon which the larvae supposedly fed. A similar situation was described by Dyson and Lloyd (1933) in sewage beds.

Although most workers assume that the microörganisms ingested by mosquitoes actually serve as food, one must be careful to distinguish this from the fortuitous ingestion of large numbers of organisms that play no important part in the nutrition of the insects. Such is the case with various species of Geotrypes which Vaternahm (1924) found did not contain an indigenous bacterial flora but only that received with the food (dung).

One of the first to advance the idea that bacteria are indispensable to growth of certain insects was Bogdanow (1906), who found that the larvae of *Calliphora vomitoria* fail to develop in the absence of microorganisms. Later (1908), Bogdanow stated that the larvae require a definite and fairly simple bacterial flora. Sterile larvae on sterile food never developed normally, although some of them reached the pupal stage. Weinland (1907), however, showed that the larvae of *Calliphora* are able to digest meat without the assistance of bacteria. Bogdanow also found that larvae of the housefly, *Musca domestica*, can be bred on starch paste or gelatin, but only in the presence of molds and bacteria. Wollman (1921) reported, however, that microbe-free cultures of flies can be maintained indefinitely, as can also similar cultures of the moth *Galleria melonella*. The work of Glaser (1924) showed that the growing larvae of flies were dependent on certain accessory growth factors which may be obtained from bacteria and yeasts, but that microorganisms and their activities are not absolutely essential to the normal growth, development and longevity of the flies. Later (1938) he developed a method whereby houseflies may be raised in sterile culture, free from microorganisms. Baumberger (1919) reported that the larvae of the fly *Desmometopam-nigrum* Zett. are probably always dependent on microorganisms and that the larvae of the housefly very probably feed on microorganisms. Trypetidae larvae can develop only when microorganisms are present, according to Stammer (1929).

#### *Yeasts and molds as food for insects*

Although bacteria alone may serve as food, considerable work has been done on the utilization of yeasts and molds as food, either one alone or together with bacteria.

In 1913 Guyénot reported, in one of a series of notes, that bacteria-free larvae of *Drosophila ampelophila* Loew. may breed entirely on yeast. Under natural conditions the larvae feed principally on yeast and other microorganisms; and the absence of microorganisms renders certain foods unsuitable. (See also Guyénot, 1917.) Northrop (1917) observed that the number of flies may be increased by the addition of banana, casein, or sugar

to the yeast. Loeb and Northrop (1917) went a step farther and showed that while the larvae of *Drosophila* cannot grow on glucose agar unless yeast is added, the imago can live well on glucose agar alone. Baumberger (1917) maintained that the insect depends on yeast for its protein.

Later, in a very thorough report on a nutritional study of insects with special reference to microorganisms and their substrates, Baumberger (1919) clarified the situation with respect to *Drosophila melanogaster*. Sterile larvae grow rapidly on sterile food but die before pupating. Decaying fruit is not the food for *Drosophila* but merely a substrate for yeast cells, although the fruit also has some additional nutritive value. Further, the larvae grow on dead as well as on living yeast. Other microorganisms (bacteria and molds) are also suitable food, but yeast is a more complete food. In general, the use of microorganisms as food is widespread among insects. According to Baumberger, the feeding habits of insects may be grouped into three classes, as follows:

1. Ingestion of microorganisms with substrate, e.g., *Drosophila*, *Musca*, *Sciara*, worker termites.
2. Feeding directly on microorganisms, e.g., tree crickets, many adult Diptera.
3. Preparation, by insects, of a substrate for the development of microorganisms, e.g., leafcutting ants, termites, ambrosia beetles.

As indicated by the third class of feeding habits, the higher fungi present many interesting relationships with insects. One of the early observed types is exhibited by the fungus-growing termites and ambrosia beetles. The carefully nurtured and "cultivated" fungus apparently furnishes these insects an ample supply of food. This relationship has been well described by Hingston (1929) who, in speaking of the ant *Atta sexdens* of Brazil, states: "The ants . . . ascend the foliage, cut pieces from the leaves and carry them back to their nest. . . . They cut them up into fragments, mould the fragments into sponge-like masses which will serve them as underground gardens. On these gardens they grow a fungus, a small white mushroom-like type of vegeta-

tion, on which the ants themselves feed and which they supply to the young ants in the nest. Each species of leaf-cutting ant grows its own particular species of fungus, and none but this particular species of fungus is allowed to grow in the nest." This author goes further to describe just how these gardens are planted and fertilized by the ants.

According to Buchner (1928), the fragments of wood bitten off by bark beetles undergo digestion in the insects' digestive tract. However, the wood is first transformed from a poor substrate into a rich food by a fungus. Each species cultivates a specific fungus. There is a dense outgrowth of fungus on the walls of the burrows of the bark beetles, and in the case of one (*Sirex*) there are special organs which harbor the fungi. Another type of symbiotic feeding on wood and similar substances is presented by the beetle *Anobium paniceum*, which has special appendages of the mid-intestine containing Saccharomycetes in their cells (Escherich, 1900; Buchner, 1921, 1928; Heitz, 1927).

Besides participating in the actual breaking down of the food in the digestive tract, bacteria may exert other influences on the digestive processes. As pointed out by Wigglesworth (1927) even such a factor as the acidity commonly observable in the crop of the cockroach is not a physiologic constant but is dependent on bacterial action. Perhaps more important, however, is the ability of microorganisms to synthesize certain accessory substances which aid in the metabolism of the insect. In many cases such possibilities exist but more experimentation is needed to clarify the matter. For instance, Zabinski (1928) observed that *Blattella germanica* synthesizes tryptophane, but was unable to decide whether this is or is not produced by the activities of symbiotic microorganisms.

Portier (1919) was one of the first to suggest that the source of vitamins for the individual insect is the intracellular organisms it possesses. Wollman (1926) probably overlooked this possibility when he claimed that cockroaches (*Blattella germanica*) may dispense with vitamins. Hobson (1933) supports Portier's hypothesis with his work on the nutrition of blow-fly larvae. He found that these larvae were unable to develop aseptically on



sterile blood owing to the lack of growth factors of the vitamin B type. The presence of bacteria improved growth, and yeast autolysate allowed the larvae to grow at a normal rate. Later on (1935), he reported that the natural flora must supply the necessary vitamins and that larvae grow readily on blood inoculated with pure cultures of various bacilli isolated from the intestine and from blown meat. *Bact. coli* proved equally effective in these experiments. Observations of Wigglesworth (1936) on *Rhodnius prolixus* Stal. support the view that symbiotic organisms in exclusively blood-sucking insects provide an endogenous source of vitamins. (See also Koch, 1933; Trager and Subbarow, 1938.)

Though not of a strictly nutritional nature, a phenomenon first reported by Atkin and Bacot (1917) and Bacot (1917) should be mentioned here. These workers found that in experiments on mosquito eggs (*Stegomyia fasciata*), the greatest stimulus to hatching is the introduction, into their environment, of living yeasts or bacteria. The stimulus produced by killed cultures of bacteria and sterile watery extract of brewer's yeast was more feeble, many of the resistant eggs failing to hatch. These, when treated with living cultures of bacteria such as *Bact. coli*, never failed to hatch. Sterile filtrates of bacteria were less effective than killed cultures. The methods of experimentation were very simple. Different species of living bacteria were introduced into tubes of sterile media, such as peptone water, in which the eggs had been lying dormant for 11 to 15, and in some cases 39 days. Upon inoculation with the bacteria all eggs hatched within 18 hours. Atkin and Bacot explain this phenomenon by supposing that the stimulus is of the nature of a "scent" which penetrates to the larvae lying dormant within the egg shells, causing them to make vigorous movements which result in the uncapping of the egg. Some of the differences of opinion concerning this phenomenon were reconciled by Rozeboom (1934), who found that a great deal depends on the age and condition of the egg. Of 240 old, dry eggs only four hatched in sterile media, whereas 204 hatched within the two days following inoculation of the media. Of fresh, moist eggs, 35 per cent hatched

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in distilled water, 51 per cent in sterile, filtered breeding water, and 82 per cent in water contaminated with bacteria. (See also Hinman, 1930, 1932.)

It seems that the ingestion of bacteria and yeasts by insects is of sanitary significance for man, and also may be important for maintaining the insect species. This is emphasized when we remember that the larvae of mosquitoes feed greedily on microorganisms. It is such biologic relationships as these that serve as an incentive for a more thorough experimental penetration of this field.

From the above discussion, we may conclude that insect larvae can be reared on sterile media if they are supplied with all the necessary food factors. As stated by Wigglesworth (1939, p. 286), "If these are deficient, infection with microorganisms (in the case of *Drosophila*, particularly the introduction of yeasts) improves the rate of growth. Sterile *Lucilia* larvae will grow on beef muscle; they fail to grow on guinea pig muscle; but if this is infected with *Bacillus coli* or if a yeast extract is added to it, normal growth takes place. . . . In these cases there is little doubt that the microorganisms are synthesizing the necessary vitamins of the 'B' group."

#### *Microorganisms and the physiology of insect digestion*

Considerable evidence has been advanced that microorganisms may play a greater rôle in the nutrition of insects than merely serving as food. The physiologist is far from knowing just how great a part microorganisms play in the digestion of food materials in the digestive tract. A few interesting examples showing the importance of microorganisms in this respect stand out.

The best known examples are the intestinal flagellates which take an active part in the digestion of cellulose in the gut of the termite (Cleveland, 1924 to 1928), and in the wood-feeding roach *Cryptocercus* (Cleveland, 1924). If, for instance, as Hegner (1938) relates, the termite, *Termopsis augusticollis*, which possesses four different types of flagellates, is kept in pure oxygen for 72 hours at atmospheric pressure, all the protozoa are destroyed but the termite is unharmed. When returned to normal

conditions the termite will eat wood but is unable to digest it and starves. If reinoculated with the flagellates, the termite can again digest the wood and live its normal life. There are other microorganisms associated with the flagellates of termites (Cleveland, 1928; Kirby, 1937), but whether they influence the symbiotic relationship in any way is not clear. Some workers have differed in their interpretation of this phenomenon in wood-eating insects. It has been claimed (Pierantoni, 1936) that the protozoa themselves contain the bacteria which in turn break down the cellulose. Mansour and Mansour-Bek (1934) state that wood-eating insects with organisms living in the intestinal lumen (as in termites and Lamellicorn larvae) use the organisms directly as food and derive no digestive help from them. These authors do not accept the view that such insects live on the products of cellulose, split by the microorganisms they harbor.

Besides protozoa, yeasts and molds are also thought to influence the digestive processes of insects, producing enzymes which are ordinarily lacking in the digestive system. On the other hand, investigators have found that the higher fungi play no rôle in the process of digestion in many insects. Brown (1928) has pointed out that extreme care must be exercised in work on the digestive enzymes of insects to exclude those produced by microorganisms, especially by bacteria. Mahdihassan (1935), in his studies on the "symbiotes" of scale insects, suggests that the presence of yeast-like forms of "symbiotes," as opposed to bacterial forms, is physiologically associated with the excessive formation of sugars. Such insects may either excrete the sugars as honey dew or further transform them into waxes or into lac.

Petri (1905) was one of the earliest to assign to the bacteria a definite digestive rôle. The bacteria constantly present in the gastric ceca of the olive fruit fly (*Dacus oleae*) were found to produce lipase. It is suggested that the activity of the bacteria in the digestion of fats must be very important for the larva which feeds on the olive, a fruit rich in fats. In a later paper (1910), he asserts that partial digestion of the oil might be possible without the aid of bacteria, since many larvae living on seeds rich in oil do not possess intestinal bacteria. Bogdanow

(1906) believed that the formation of ammonia during larval development of *Calliphora vomitoria* is not a characteristic of protein digestion by the larvae but probably a result of bacterial activity. Weinland (1907), on the other hand, insisted that the ammonia is the result of larval metabolism. Wollman (1911, 1921) indicates that Weinland was mistaken; no ammonia is produced by sterile larvae and its production is due to microorganisms. (See also Hobson, 1932.) Weinland (1908) observed further that bacteria take no part in the process of fat formation in the larvae. Guyénot (1906, 1907) found that muscid larvae (mostly those of *Lucilia*) are unable to produce any digestive ferments which liquefy meat. He believed that this is accomplished by bacteria. In contradistinction, Wollman (1921) claims that aseptically bred larvae liquefy gelatin, which indicates that they produce some proteolytic ferments.

Portier (1911) claims that leaf-mining larvae of *Nepticula malella* and *Gracilaria syringella* live under sterile conditions and do not harbor any microorganisms in their bodies. On the other hand, the normal leaf-feeding larva of the silkworm, *Bombyx mori*, has its digestive tube populated with microorganisms, some of which destroy the wall of the leaf cell, while others thrive on its contents. The larva uses the cell contents directly as food. Glaser (1925), however, reared large numbers of silkworms and rarely found many bacteria in the digestive system of normal worms. Where bacteria became numerically high, the worms ailed and died. Hering (1926) criticized some of the views of Portier, stating that up to that time no true "symbionts" were known in leaf-miners. Werner (1926) found that the digestive tract of the larva of *Potosia cuprea* Farb. has a very rich microflora able to cause the fermentation of cellulose. A specific bacterium was isolated and named *Bacillus cellulosam fermentans*. Schütte (1921) found that cellulose is digested by the larva of *Hydromyza livens* F., but apparently without the aid of bacteria.

It should be remembered, when one is considering the rôle of cellulose-fermenting bacteria in the nutrition of insects, that in most phytophagous insects the food passes through the gut so rapidly (Wigglesworth, 1939, p. 271) that no great amount of

fermentation is likely to take place. The breakdown of cellulose by bacteria is usually too slow a process to be initiated and completed in the few hours during which food remains in the gut. On the other hand, cellulose-splitting bacteria are often associated with the food ingested by insects and for this reason cannot be completely ignored. Furthermore, certain insects, such as the Lamellicorn larva, possess a "fermentation chamber" which is probably used for such purposes (Mingazzini, 1889a, b). Ripper (1930) points out that in the larvae of *Dorcus* and *Osmoderma*, there is no evidence of cellulose breakdown even though the fermentation chamber is full of bacteria.

In 1919 Roubaud asserted that adult tsetse flies were exclusively hemophagous. The blood ingested by the flies was digested only in the middle section of the intestine where the epithelial cells include symbiotic organisms. According to Roubaud, these organisms play an important part in the digestion of blood. Wigglesworth (1929) states, however, that there is no evidence that these organisms play any part in the digestion of the blood.

The true function of the bacteria living in the cecal pouches of various Hemiptera is not altogether clear. Glasgow (1914) and Kuskop (1924) both believe that the bacteria contained in the cecal pouches exclude foreign bacteria altogether. Elson (1937) believes that, since bacteria are absent in the predatory and semi-predatory species of Hemiptera, the presence or absence of microorganisms bears an important relationship to the food habits of the insect. Wigglesworth (1939) states that there is no evidence that bacteria in the ceca of the Hemiptera play any part in digestion.

#### INTRACELLULAR FLORA OF INSECTS

Perhaps the most interesting of all the biologic relationships between microorganisms and insects are those of intracellular parasitism and symbiosis. Within the tissue cells of many arthropods may be found apparently living, non-pathogenic organisms. In some cases, such as the rickettsiae pathogenic for

lice, the intracellular organisms may exert a deleterious influence on the host, but usually they are not only harmless but may be distinctly symbiotic. For this reason such microorganisms have been termed "symbionts" or "symbiotes"<sup>2</sup> by some writers with the assumption that they live in a symbiotic relationship with the arthropod host. This has been the viewpoint from which Buchner (1921b, 1930) has ably reviewed the field. Other excellent reviews of the subject have been presented by Glaser (1930c) and Paillot (1933), and it is unnecessary to repeat their detailed accounts here. Only the high points of the subject will be discussed to indicate the very intimate biologic relationships between microorganisms and insects. It should be mentioned that similar associations exist between microorganisms and arachnids. (See Mudrow, 1932; Gregson, 1938; Trager, 1939.)

Among the earliest to recognize the presence of intracellular "organisms" in the normal eggs and body tissues of insects was Blochmann (1886). He noted that bacteria-like bodies were present both in the eggs and follicular membranes of ants and wasps, and suspected that these forms were bacteria. In the following two years Blochmann (1887, 1888) indicated more confidence as to the nature of these bodies, which were also found in the fat tissue and eggs of *Periplaneta orientalis* and *Blatta germanica*. His contention that they were bacteria was based on their reaction to various reagents and stains, their multiplication by fission, and their method of infection through the eggs. His attempts to cultivate the "organisms" in beef-peptone-gelatin-agar media were unsuccessful. During the next few years Wheeler (1889), Cholodkowsky (1891), and Heymons (1895) confirmed these observations.

The aphids were among the first insects studied with respect to their intracellular "organisms." As early as 1850 Leydig

<sup>2</sup> Both of these terms are used interchangeably throughout the literature. Although "symbiont" is considered by some writers to be a misnomer, it is used more frequently perhaps than the term "symbiote," from the Greek word for "partner" or "companion."

observed certain organs in aphids which have subsequently been called "symbiotic organs," "pseudovitellus," "green body," and "mycetome" or "mycetom." The cells making up these organs are usually known as "mycetocytes" or "bacteriocytes" and it is in these that the intracellular forms are usually located. Following Leydig's report, the nature, origin and development of these organs were described by many workers including Huxley (1858), Balbiani (1866 to 1871), and Tannreuther (1907).

Krassilstchik (1889, 1890), however, seems to have been the first to begin to shift the emphasis from the anatomic study of the mycetome to a study of the bacteria-like forms within this organ. He named these forms "biophytic bacteria." Pierantoni (1909 to 1911) and Šulc (1910) attempted to show that the forms within the mycetocytes were living organisms related to the yeasts. It was Šulc who suggested the name "mycetom" for the "symbiotic" organ, and "mycetocyte" for the individual "symbiotic" cell. (See also Klevenhusen, 1927.)

Not only aphids but many other insects have been reported to possess mycetomes and intracellular organisms. Buchner (1912, 1921) has published excellent accounts of what he considers symbiosis in aleyrodids, coccids, aphids, psyllids, cicadellids, blattids, Cicada, Hymenoptera, Coleoptera, and Lepidoptera. An account of the intracellular inclusions and the embryologic development of the mycetome in aphids has been presented by Uichanco (1924). Mansour (1934a, b) has furnished considerable data concerning the intracellular microorganisms in coleopterous insects; and Gier (1936) has studied the structure and behavior of the intracellular "bacteroids" of roaches. (See also Blochmann, 1892; Florence, 1924.)

Interesting light-producing organs and groups of cells, comparable to the mycetomes and bacteriocytes, have been found in some insects as well as in certain other animals. According to some writers (Buchner, 1921b; Wallin, 1927) these organs are associated with luminescence in certain insects and harbor "phosphorescent" or luminiferous bacteria. These authors also cite various claims which have been made concerning the artificial cultivation of these luminous bacteria.

*Transmission of intracellular microorganisms*

One of the interesting aspects of this study is the nature of the transmission of these organisms from parent to offspring. Krasilstehik, in his reports on the aphid, observed not only that they are transmitted directly from one generation to the next, but also that they are present in every representative of the species, including the embryo. Uichanco (1924) traced the path of transmission of the symbionts from the follicular epithelium of the parent to the definitely formed mycetome in the offspring. Mansour (1934a), working with certain Bostrychid beetles, reported the transmission of microorganisms from one generation of the host to the next thus: The microorganisms from the mycetomes invade the lobes of the testes, multiply, and mix with the sperm. The microorganisms then pass with the sperm during copulation into the bursa copulatrix of the female. From this region they pass through the micropyle of the fully formed egg during its passage to the outside, and thus the infection is accomplished. (See also Mansour, 1930.) Gier (1936) in working with the "bacteroids" of roaches found that the number of "bacteroids" between the oöcyte membrane and follicle cells increases until there is a uniform layer two or three organisms thick. Before the egg is oviposited, the original oöcyte membrane breaks down and permits the "bacteroids" to enter the cytoplasm. This introduces them into the embryo and a transmission from one generation to the next has been accomplished. No doubt many other new and interesting modes of transmission of the intracellular microorganisms in insects remain to be discovered.

*Nature and rôle of intracellular organisms*

In reading the literature on this subject, one becomes aware of the feeling of uncertainty expressed by various writers as to the true nature of these cellular inclusions. Are they actually living microorganisms? If they are, do they fall into the category of bacteria or of yeasts, or do they belong to that group of microorganisms known as *Rickettsia*?

Some workers (Cuenot, 1896; Henneguy, 1904) have interpreted these intracellular inclusions as mitochondria or as waste



products. The application of various methods of differentiation has shown that mitochondria can usually be distinguished from microorganisms (Cowdry and Olitsky, 1922; Cowdry, 1923). On this basis most workers consider the forms to be living organisms. If they were easily cultivable on artificial media such inclusions would immediately be removed from the category of mitochondria or of waste products. In a large majority of cases, however, very little or no success has been had in the attempts to cultivate these agents artificially.

Two investigators, Pierantoni (1910) and Peklo (see Uichanco, 1924), have reported successful cultivation of the symbionts in aphids. As Uichanco (1924) points out, however, neither of these workers "appears to have furnished adequate evidence to prove the identity of their artificially obtained microorganisms with the 'microorganisms' of the aphid 'mycetocytes'." On the other hand, the symbionts of *Pulvinaria innumerabilis* Rath. have been cultivated by Brues and Glaser (1921). In this case the microorganisms are found in the blood and adipose tissue.

Hertig (1921) was unable to culture the "bacteroids" of the Blattidae and decided that the organism (*Bacillus cuenoti*) cultured earlier by Mercier (1907) from these insects was a contaminant. Glaser (1930a) has reported the cultivation of the microorganisms from the American roach, *Periplaneta americana*. To the diphtheroidal forms isolated he gave the name *Corynebacterium periplanetae*, var. *americana*. In the same year Glaser (1930b) cultivated from the German roach, *Blattella germanica*, an organism he designated as *Corynebacterium blattellae*. On the other hand, Gier (1936) indicates that only negative results were obtained in his attempts to cultivate the "bacteroids" from roaches. In a personal communication, Gier asserts that he made hundreds of attempts to cultivate these forms on many kinds of media, but always without success. He believes that Glaser's diphtheroids are slow-growing contaminants of rather unusual character. He holds the results of Bode (1936) as well as those of Mercier (1907) to be due to poor techniques. Gier was unable to get any signs of growth on chick

chorio-allantoic membranes or in amniotic fluid either with the living chick or in a test tube. Glaser (personal communication) explains the failure of other workers to duplicate his results as due to faulty technique. He maintains that repeated transfers from a medium which has been inoculated with these microorganisms, but which is apparently sterile, are necessary before the growth of the organism finally appears as tiny pin-point colonies. According to Glaser, a period of slow adaptation to artificial conditions apparently is necessary. Glaser also believes that successful cultivation may depend not only on the age of the roach but on the season of the year as well. (See also Neukomm, 1927a, b.)

It is interesting to note that Gier found the "bacteroids" doubling their numbers in the embryos and young nymphs in about ten days. With the rate of increase, the numbers diminished as the animals neared maturity, and apparently decreased in adults except in the ovaries. Long-continued starvation, sublethal doses of X-rays or ultraviolet light, and the injection of crystal violet, hexylresorcinol, and metaphane brought about a decrease in the numbers of microorganisms.

In the writer's opinion, biologists should hesitate to conclude that these forms are not microorganisms simply because of the difficulties encountered in cultivating them. This fault may lie in the inadequacies of the methods and not in the nature of the organisms themselves. The possibility that many of these intracellular microorganisms are closely related to rickettsiae, a group which has not yet been thoroughly defined or given its taxonomic boundaries, should not be overlooked. How close a relation exists between rickettsiae and bacteria has been far from determined. There is already some indication of a taxonomic overlapping of these two groups. In some cases it is fairly certain that the intracellular organisms are of the nature of true bacteria, while in other instances they are definitely rickettsiae. Between the two lies an already large group of non-pathogenic intracellular forms that possess certain characteristics of both. The surveys of Cowdry (1923) and of Hertig and Wolbach (1924)

make the biologist aware of the fact that here lies a large field waiting to be explored, not only from the purely biologic standpoint but for taxonomic reasons as well.

It seems very probable that in the past much of the difficulty encountered in culturing these intracellular organisms was due to the imperfect culture methods used. Many of the early workers went no farther than to use ordinary nutrient agar or broth in their culture experiments. Modern bacteriologic techniques and media have been much improved since that time. The application, in recent years, of tissue culture and chick embryo methods in growing rickettsiae and viruses suggests the possibility of these techniques for the cultivation of the intracellular organisms of insects. Perhaps the difficulty encountered in culturing these forms depends on the extent of their dependence on the host. That is, the closer a microorganism is to being a true and absolute symbiont, the more difficult it may be to cultivate in a foreign environment. Here, indeed, is a branch of the subject that requires expansion.

When we wish to consider the true rôle of these intracellular microorganisms, we find ourselves in a sea of uncertain speculation with only a few small fragments of possible explanations. There is a trend, in much of the literature, toward considering these cellular inhabitants as true symbiotic microorganisms. Others feel that they represent some sort of non-symbiotic parasites. Some consider the bacteria-containing organs, the mycetomes, as analogous to plant galls produced by the host as a response to the irritation by a foreign inhabitant. The microorganisms have also been regarded as true parasites to which the insect has developed an immunity. (See Mansour, 1934a; Paillot, 1931.) Since the physiologic action of these organisms remains so obscure, little is known as to the exact benefit or harm to their host.

It is often quite difficult to make a definite distinction between symbiosis and parasitism. It is generally recognized that there are degrees of symbiosis, that is, symbiosis may be obligate or facultative. In the case of the intracellular organisms in insects, very little work has been done to determine whether or not the

association between these two forms is indispensable to both. It is very probable, however, that both the obligate and the facultative types of symbiosis exist between insects and their symbionts. In any case, it is sometimes difficult to imagine just what constitutes the "mutual advantage" to the two forms of life concerned. Although there are some experimental data on the benefits secured by the host through such a relationship, the evidence for the advantage to the symbionts is considerably more uncertain and hypothetical. This is indicated in a statement by Meyer (1925): "Buchner's suggestion that the intracellular organisms are benefited by being protected within the host from the drastic atmospheric influences of heat, cold, desiccation, etc., is a trifle unreasonable."

The reasons why these organisms have been generally considered to be symbiotic in nature rather than truly parasitic have been set forth by Glaser (1920) as follows:

- (a) Every individual of a species is infected.
- (b) The infection produces changes in the host cells, but these are harmless.
- (c) The infection routes and methods of localization, while different in different hosts and symbionts, follow very definite courses within a species.
- (d) The microorganisms are numerically controlled by the host, never increasing up to a point where they prove fatal.
- (e) The microorganisms within the insects obtain nourishment and protection from drastic temperature and drought conditions.

To these reasons may be added the experimental evidence presented by Aschner (1932, 1934) and Aschner and Ries (1933) who obtained results which, in their opinion, warranted the conclusion that the symbionts play an essential rôle in the life of the body louse. It was found that if the louse *Pediculus* is deprived of its symbionts by operative removal of the mycetome or through elimination of the symbionts by centrifugalisation of the egg, its powers of nutrition and reproduction are greatly impaired. Without the organisms the larvae die and, according to Aschner, it is justifiable to consider this relationship a true symbiosis.

Some workers have thought that in certain insects the close association of the symbionts with the intestinal tract indicates that the microorganisms might aid in digestion (See Buchner, 1930). There is, however, little evidence to support such a view. As stated by Wigglesworth (1939), if these are of value it appears more likely that they contribute to nutrition or metabolism. The process of reproduction in some aphids seems to be intimately associated with the activity of the symbionts. In some cases the latter are thought to synthesize protein for their host during reproductive activity. Wigglesworth also suggests the possibility that they provide accessory factors, and thus enable their host to live permanently on a restricted or highly specialized diet deficient in some respects.

In general, it appears that although in some cases symbionts may not always be essential to life, it is reasonably certain that in other cases they serve a useful purpose. (See also Ries, 1932, 1935; Aschner and Ries, 1933; Koch, 1936a, b.)

Another theory to account for the apparent harmlessness of these intracellular forms is that they have but reached a certain stage in an evolutionary process of adaptation. This, of course, has already been suggested by various writers, especially in the case of the rickettsiae associated with ticks. It is not difficult to imagine that in their initial association, these microorganisms were actually pathogenic parasites, later assuming a more or less commensal relationship until finally a definite mutualistic association was established between the host and the invaders. Certainly the association of these two forms of life must have been an extended one, especially in view of the congenital transmission of the organisms, and for the reason that most insects seem to have such an intimate connection with these forms. It might be emphasized, however, that in certain ants and in the weevil *Calandra granaria* the mycetome is present but apparently contains no symbionts. Some writers have interpreted the formation of mycetomes as the development, on the part of the host, of highly specialized organs whose sole function is that of harboring the symbionts. Such a development is certainly not beyond the range of possibility. Various types of response are known to

result from the entrance of microorganisms into the tissues of plants and animals. As stated by Wallin (1927), these responses, in general, may be both physiologic and morphologic. The presence of mycetomes and bacteriocytes could probably be cited as an example of morphologic response on the part of the host to the intracellular inhabitants. (See also Lilienstern, 1932; Mansour, 1934a.)

Because of the wide disagreement as to the true physiologic nature of these intracellular organisms, Gier (1936) sought to avoid such terms as "symbiont," "symbiosis," "parasite," and "parasitism" and the implications arising from their usage. Accordingly he designated the intracellular bodies in roaches as "bacteroids." No doubt there is considerable justification in using such a term if it is taken to mean "bacteria-like." However, care must be taken in using a designation which has already a definite meaning in soil microbiology. The term "bacteroid" was first used by Brunchort (1885) in referring to the root nodule organisms in the legume plants. As defined by Fred, Baldwin and McCoy (1932), this term is used to designate "the enlarged, frequently club-shaped or branched, vacuolated or banded forms of the root nodule bacteria, both as they occur in the nodule and in culture media." Simply because comparable forms are found associated with insects is no valid reason why these cannot be called "bacteroids." Nevertheless, the same objection may be raised in this instance as that made in the case of the root nodule bacteria by Löhnis and Smith (1916). They objected to the use of the term "bacteroid" because it is now generally recognized that these are forms of true bacteria and not plant products resembling bacteria. Similarly, when the intracellular forms in insects are more thoroughly studied, and perhaps some placed among the bacteria, they then will deserve more than the mere designation of "bacteria-like."

There is considerable danger in too great a reliance upon pathogenic characteristics in determining the taxonomic grouping of microorganisms. Many writers feel that unless an intracellular organism is pathogenic for higher animals it should not be grouped with the rickettsiae even though its morphologic characters are

compatible with such a grouping. Merely because the rickettsia-like organism, such as the one (*Rickettsia lectularia*) in the bedbug (Arkwright et al., 1921; Buchner, 1921c, 1923; Pfeiffer, 1931) is of the symbiotic or commensal rather than of the pathogenic type, such as *R. prowazeki*, is no reason why the non-pathogenic form cannot be legitimately considered a member of the *Rickettsia* group. As pointed out by Hertig and Wolbach (1924), "Bacteria botanically related, for instance the acid-fast bacteria, show comparable extremes in nature of habitat and pathogenicity." The taxonomic characteristics of the rickettsiae as a group are still so imperfectly defined that it seems very likely that its boundaries will be considerably modified before a definite systematic basis is established. There are already those who feel that the term *Rickettsia* should be reserved as a generic name of a particular group of these organisms, such as *R. prowazeki* and those related to it (Macchiavello, 1938). Other types of rickettsiae and rickettsia-like organisms may then be assigned other generic names under the proper family (Rickettsiaceae?). An example of this is the generic name "*Wolbachia*" suggested by Hertig (1936) for the genus of which *Wolbachia pipientis* is the type species.

Kligler and Aschner (1931) give an excellent statement of this phase of the problem: "... further advances in our knowledge of this group of microbes depends on a greater familiarity with the flora of insect parasites in general, and, more particularly, with the group of organisms having the general characteristics of *Rickettsia*. . . Knowledge of the cultural properties of the non-pathogenic members of this group of microbes may aid in the study of the pathogenic members of this group."

#### OTHER BIOLOGIC RELATIONSHIPS BETWEEN MICROÖRGANISMS AND INSECTS

One cannot hope to include all the biologic relationships between microörganisms and insects within the limits of this review. For this reason, the relatively large field of insect diseases has not been included. It would be inexcusable, however, not to mention this subject as one of the important biologic relationships

existing between these different forms of life. The use of pathogenic agents in controlling insect plagues has been attempted with varying degrees of success. (See Sweetman, 1936.) It is evident that progress in our knowledge of disseminating or controlling the diseases of insects has only begun. This is likewise true of the study of the immunologic aspects of insect diseases. Huff (1940) has included this phase of the subject in a recent review of immunity in invertebrates. Linked with this is the possibility of a better understanding of the principles of epidemiology through the observation of diseases in large insect populations.

Many more examples of the biologic relationships between microorganisms and insects could be added. Pospelov (1926) believes that symbiotic organisms in the fat-cells of certain Lepidoptera serve as food for the developing genital glands and other imaginal tissues. Mahdihassan (1928 to 1935) has been able indirectly to differentiate species of coccids by examining blood smears containing their symbiotic microorganisms which show morphologically distinct forms dependent on the species of insect harboring them. Although according to Snodgrass (1935, p. 50) chitin is not attacked by mammalian digestive enzymes, it is, according to Benecke (1905) broken down by *Bacillus chitinovor*, which, as stated by Snodgrass, may be the agent of its decomposition in nature. The relation of the phytotoxic secretion of *Pseudococcus brevipes* to its symbiont has been studied by Carter (1935, 1936). Black (1939) found that the juices of insect vectors inhibit the infectivity of tobacco-mosaic virus for Early Golden Cluster Beans. Bacteria have been shown to be able to change the eye color in *Drosophila* flies (Tatum, 1939). The change from white to brown color in the eyes is caused by a "hormone" produced by the microorganisms. These and many more examples indicate the various interesting aspects of this field which could be pursued to good advantage.

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## THE MENINGOCOCCUS (NEISSERIA INTRACELLULARIS)

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During the last decade there have been some interesting developments in our knowledge of the meningococcus. The purpose of this paper is to summarize these findings. It is necessary first to give a brief survey of our older knowledge of this microorganism in order to give perspective to our newer information.

This is not a monograph and is by no means comparable to the classic report of Elser and Huntoon (32) in 1909, or to the intensive review made by Murray (81) in 1929. It might in one sense be termed a "progress report" since much work is being done at present that may later clarify some of our greatest problems.

No attempt will be made to give a complete bibliography of all the work that has been done either during this decade or before, but for the sake of brevity reference will be made only to those papers that illustrate the facts under discussion.

This paper is written from a bacteriological point of view. Clinical and therapeutic discussions are not included as such, but occur only as bacteriological findings bear directly upon them.

### HISTORY OF OUR EARLY KNOWLEDGE OF THE MENINGOCOCCUS

First recognition of meningococcus meningitis as a definite clinical entity goes back to the early days of the 19th century (101, 25), but it was to be many years before the causative organism became known.

In his *Handbook of Geographical and Historical Pathology*, published in 1886, Hirsch (56) devoted an interesting chapter to epidemic cerebrospinal meningitis. He discussed the epidemiology in great detail, described the occurrence of four major

epidemic cycles during the preceding fifty years, told of its seasonal preference, and mentioned the effect of fatigue and lowered resistance on susceptibility. He was one of the first to point out that the infection is especially apt to occur in children and in soldiers, and that the disease has a tendency to occur in waves, with a peak of incidence occurring about every ten years. He concluded that the disease must undoubtedly be of an infective nature, "transportable" rather than "contagious."

It was in the next year, 1887, that Weichselbaum (103) published his description of the meningococcus as recovered from six cases of acute cerebrospinal meningitis, and named it *Diplococcus intracellularis meningitidis*. Weichselbaum's description of the meningococcus is so accurate that there has been no occasion to dispute any of his findings in the years since.

During the following decade there appeared many case reports in which Weichselbaum's observations were confirmed. In many instances the meningococcus was merely seen in spinal fluid and in meningeal exudate at autopsy (70, 71, 97). In other instances it was actually cultivated (44, 55, 59, 66). In 1898, Councilman, Mallory and Wright (24) demonstrated the meningococcus in 31 out of 34 cases of meningitis. These reports securely established the organism as the cause of acute cerebrospinal meningitis.

Many fundamental facts about the meningococcus were established by these early students. They described its occurrence in pairs with flattened adjacent sides,—“two hemispheres separated by an unstained interval.” The organism was apt to occur within the leucocytes in spinal fluid, but it might occur singly, in pairs, or in tetrads in cultures. It was described as being consistently gram-negative although the individual cells varied in the intensity of their staining. They found that it grew best on solid media, that it did not usually grow at room temperature, and that it was necessary to make fresh transplants at least every 2 days on the Loeffler blood-serum medium which they found best suited to it among the media then available. Weichselbaum found fresh cultures pathogenic for guinea pigs and rabbits, when given intraperitoneally, but Councilman,

Mallory and Wright were struck by the feeble pathogenicity of their strains for these animals and reported successful infection only in a goat.

The first decade of the 20th century brought further careful work on the meningococcus. A lively controversy was waged at one time by Albrecht and Ghon (1), on the one hand, and Jaeger (61, 62) on the other, concerning the gram-staining properties of this microörganism. Albrecht and Ghon felt that Jaeger's criterion of a meningococcus was too broad, and they collected evidence to show that the organism is definite in morphology and consistently gram-negative. The evidence presented by Albrecht and Ghon was further supplemented by Elser's report of 135 cases (31).

Flexner (37) studied especially the biology and pathogenicity of the meningococcus. He showed that virulence deteriorates rapidly and may disappear within a few days, and that "endotoxins" may be a greater factor in the death of animals than multiplication of the bacteria when large inocula are used. Even freshly isolated cultures were found to be of uncertain virulence. He produced a true meningitis in monkeys (38) by intraspinal inoculation, but preferred young guinea pigs, injected intraperitoneally, for experimental work. He paid considerable attention to the action of salt and believed the usual "physiological salt solution" to be very toxic for meningococci.

Wollstein (104) called attention to the close relationship of the meningococcus to the gonococcus. She stated that aside from the pathological effects in man, the chief difference between these organisms is cultural, and that demonstrable antibodies are largely common to both—a relationship which does not exist among other species in the genus *Neisseria* and which is generally overlooked. In 1909 the classic studies of Elser and Huntoon were published (32). In the same year, Arkwright (6) demonstrated that epidemic and sporadic strains were alike culturally and biochemically. He found some evidence of serological groups, but made no attempt at classification on this basis.

The period of classification really began in 1909, when Dopter

(27) found that the strains of meningococci studied by him fell into two serological groups. He called these "meningococci" and "parameningococci." Wollstein (105) made an intensive study of Dopter's strains and others, confirming his observations as to the existence of two groups indistinguishable except by serological methods. Furthermore, she found these serological differences to be supported by protection experiments with guinea pigs and monkeys, which were indicative but not absolute proof. It was about this time that Dopter and Pauron (28, 29) found that their parameningococci could be further divided into at least three subgroups, which they designated as  $\alpha$ ,  $\beta$ , and  $\gamma$ . This last very important finding seems to have been lost sight of in the years since.

#### THE WORLD WAR EPIDEMICS

The World War period of 1914-18 brought serious epidemics of meningococcus meningitis to the army camps of all nations involved. A tremendous amount of work was done on the meningococcus during this time and the literature of the period is voluminous. Much was done on the question of classification and on the relation of carrier strains to case strains, as ground work for control and therapy.

A confirmation of the existence of two main groups of meningococci resulted from the studies of most of the British workers. Griffith (51, 52), Scott (94), and Andrewes (5) designated these as Group I and Group II. Ellis (30) called them Type I and Type II. Arkwright (7) referred merely to "two main groups." It is interesting to note that Fildes (36), whose work was done several years later, realizing that the use of the terms "Type" and "Group" interchangeably was causing confusion, used the expressions "Super Group I" and "Super Group II" in order that his recognition of 2 groups might be perfectly clear.

A further division of these two groups into 4 types (I, II, III, IV), on the basis of agglutinin-absorption, was made by Gordon and Murray (46) in 1915, and a large number were typed on that basis in 1917-18 by Tulloch (99, 100) in Britain and in 1918-20 by Hitchens and Robinson (58), and by Butterfield and

Neill (21) in this country. During this same period the French workers, Nicolle, Debains and Jouan (83), described 4 types: 2 principal types (A and B) and 2 rare types (C and D). In the United States, Evans (33) classified meningococci according to their tropin reactions. She found 4 distinct groups, and a fifth group which was related to the others.

The relation of these different classifications to each other is shown in table 1. Some of the material in this table is taken from one published in 1929 by Murray in his classic monograph.

From this comparison of the different classifications that developed during this World War period, we can see that there

TABLE 1  
*Correlation of various classifications of meningococci*

DOPTER (26)	NICOLLE, DEBAINS, AND JOUAN (83)	GRIFFITH AND SCOTT (51, 52, 94)	GORDON AND MURRAY (46)	EVANS (33)
Meningococcus	A	I	I III	R
Parameningococcus	B	II	II IV	S-U Z
	C D			T?

is a general agreement that meningococci fall into two main groups. The further division of these two groups into 4 types, as done by Gordon and Murray with the absorption of agglutinin technique, is laborious and requires sera made from very carefully chosen strains. As Fildes (36) pointed out, "Types I and III and Types II and IV of Gordon and Murray are so closely related that a change in standards could result in a change of type. The classification of meningococci is real and is not dependent upon arbitrary selection of standards. But selecting standards for determining the subgroups is arbitrary and may lead to much confusion if one and the same standard is not maintained."

In spite of its complications this 4-type classification of Gordon and Murray obtained great popularity at once and has been used



in nearly all English-speaking and South American countries from then to the present day. On the European continent, the French Groups A and B are more generally recognized.

There has apparently never been any uniformity in the terms used to designate the serological groups of the meningococcus by those accepting the Gordon and Murray classification. In their original report, Gordon and Murray (46) referred to Groups I, II, III and IV, using the term "Group" (capitalized) and with Roman numerals. In subsequent papers, Gordon (47) has referred to Types 1, 2, 3 and 4, using "Type" instead of "Group," and Arabic numerals. In one paper (48) he uses both Types 1, 2, 3 and 4 and Types I, II, III and IV. Most of those who used Gordon and Murray's classification in typing referred to Types I, II, III and IV and thus that form of designation is the one that has come into common use.

It was not only in classification that this activity in the study of the meningococcus was found. Extensive surveys of carriers were made in the effort to control the spread of the infection. These resulted in studies of the biology, physiology, and cultural features of the organism, as the nasopharyngeal and spinal fluid strains were compared.

Much attention was given, too, to the study of therapeutic serum. Jochman (64) had made the first antimeningococcus serum in 1906, and Flexner and Jobling had established its value in therapy (41, 42) in time for such serum to be used in treating patients during the severe army camp epidemics. During these epidemics it was found that all lots of serum were not equally effective. Much time was therefore given to the study of toxicity, pathogenicity, and antigenicity of the meningococcus, and to preparation of antiserum and its standardization.

Jochman (64) had standardized his first antiserum by a method involving the protection of guinea pigs and mice. Flexner (39) had used guinea pigs and monkeys for this purpose at first; but he did not consider such a method of standardization satisfactory because the meningococcus cultures were of such low virulence for these animals that the very large doses used seemed to kill from toxicity rather than by invasion. Flexner and Jobling

later used a complement-fixation test (40) and the neutralization of an autolysate in guinea pigs. Kraus and Doerr (67) and Dopter (26) had advocated protection tests against endotoxin as a basis for standardization.

Houston and Rankin (60) first studied phagocytosis with meningococci, and Neufeld in 1908 (82) was apparently the first to apply this technique to standardization of meningococcus serum. Jobling (63) considered specific phagocytosis the best method of standardizing antimeningococcus serum. He used the methods of Neufeld (82) and of Leishman (72). Kraus and Baecher (68) advocated this test also. A most interesting note in those early days was Ruppel's (92) report of a meningococcus culture of such virulence that 1 ml. of a 1:1,000,000 dilution was uniformly fatal to mice; this he used in standardizing serum. Here we seem to have the beginning of the mouse-protection test, as early as 1906.

All of these studies were made before serological differences among meningococci were recognized, and therefore before it could be known that many of these tests, such as complement fixation, were tests not only for all meningococci but for gonococci and some other members of the genus *Neisseria* as well. With the recognition of serological differences among meningococci, it was realized that not even tests that indicated the meningococcus alone would suffice for serum testing.

With the numerous epidemics of meningococcus meningitis that followed the World War came realization of the need of a uniform method of standardizing serum, one which would take the differences among meningococci into account. Amoss (2) was a leader in this field in this country. He used a number of strains of meningococcus in immunizing horses, employing cells and their autolysates, and tested the resulting sera for agglutination, phagocytosis, complement fixation, and protective value in young guinea pigs. He considered phagocytosis and complement fixation to be inadequate because they did not distinguish between types. Protection tests were found to give irregular results because of the low virulence of his cultures. He (3, 4) decided that agglutination gave the best idea of the value of the

serum and of its polyvalency. An agglutination test was finally adopted officially by the United States Public Health Service, and a standard technique for its performance was worked out by McCoy and Leake (75) in 1918.

In 1916 Hitchens and Robinson (57) described a mouse protection method of standardizing serum which they felt was a truer measure of therapeutic effectiveness of serum than agglutination. In 1920 (33) Evans described a technique based on the specific tropin groups of her classification, and their specific antibodies.

Meanwhile, in England, Gordon had become convinced that the "endotoxins" of the meningococcus were very important in the pathogenesis of this microorganism and that serum, in order to be useful, should contain "antiendotoxin." He described a method (49) in 1918 for determining the antiendotoxic content of the horse sera, using mice, and studied means of producing sera with higher antiendotoxin content. Gordon found considerable clinical evidence to support his views, but his test was not simple to perform and results were apt to be irregular. The success of the mouse protective test of Hitchens and Robinson depended upon high virulence of the meningococci used and cultures with this quality were not generally available. The technique of Evans' bacteriotropin test was very exacting. Thus the agglutination test was soon generally used. Those advocating it did not think it to be an ideal method but considered that it at least indicated the presence of antibodies and the polyvalency of the serum.

It will not be profitable to refer in further detail to the vast volume of literature of this World War period, as it would only leave the reader in a state of confusion. A tremendous amount of work was done, and the resulting literature was both voluminous and confusing. Then, with the coming of peace, meningococcus meningitis suddenly ceased to be so common. Cases of meningitis occurred only sporadically; meningococci isolated from them were more difficult to type. Both experimental work and preparation of antiserum were done with old strains carried over from the former period of intensive study.

## INTEREPIDEMIC YEARS

From this World War period we emerged with a belief that our skirmishes with the meningococcus had led to advances along at least three lines: Meningococci were classified into 4 serological divisions, antiserum was polyvalent, and it was conveniently standardized by an agglutination test. During 1923 the first edition of Bergey's Manual (8) was published and the meningococcus appeared as *Neisseria intracellularis*, by which name it has since been known.

In this country antiserum was made in a number of localities by immunizing horses with the available cultures representing the 4 types of Gordon and Murray, and these antisera were standardized against a "control" serum by an agglutination test, using standard type strains as antigens. Some laboratories produced antiserum by immunizing horses with many strains, on the basis of Amoss' theory (2) that a great variation occurred among strains, and to be truly polyvalent many should be represented. Other laboratories used no more than 4 to 6 strains, on the basis of Wadsworth's theory (102) that a few well-chosen strains of good antigenicity were adequate. Apparently satisfactory antiserum was prepared by both methods.

Most of the actual and experimental work of this period was done, necessarily, with old strains of meningococci. They were, for the most part, both avirulent and non-specific. There was much "crossing" between types in agglutination, and good typing sera were difficult to prepare. Protection tests, using small animals, were thought to be out of the question. Bacteriologists who had worked much with the meningococcus recognized that the cultures had changed culturally, serologically, and antigenically. Many present workers can remember the ringing tones with which the late Doctor Krumwiede denounced these "degenerated meningococci," from the floor of bacteriological meetings. Occasionally promising new strains from some of the few cases of meningococcus infection would be obtained, but by the time studies with them were well under way, virulence was gone. There was, on the whole, not very much lively

interest in the meningococcus during this time. There were no extensive or severe epidemics. It was generally felt that serum therapy in meningococcus meningitis was well established and on a satisfactory basis. Plenty of antiserum was being made and standardized. There seemed no special cause for concern.

Suddenly, reported cases of meningococcus meningitis leaped in number from 1700 in 1926 to 3000 in 1927, and to 10,551 by 1929. Mortality was very high—as much as 70 to 80 per cent in some localities—and serum therapy seemed entirely inadequate. Something was evidently the matter. Why should serum therapy, which had been so successful during the years immediately after its introduction, be so useless now? Were the invading strains of the meningococcus different? Was serum being made by the wrong methods? Was the method of standardization at fault?

#### EPIDEMIC PERIODS OF 1928-30 AND 1935-36

Again the meningococcus became the subject of intensive interest. For a time there was abundant material for study. Literally, hundreds of fresh new cultures of meningococci were available.

Typing sera were prepared in rabbits, using the old standard type cultures representative of the Gordon classification. These cultures had “spread” antigenically, and typing was not always easy. By far the greatest number of the new strains fell into Types I and III, though a few were of Type II, and in the Chicago epidemic Type IV was abundant. It was realized very early that typing was easier if done soon after isolation of the strain, and that antigenicity soon began to “spread” and cross-agglutination became marked.

The Type IV cultures were easily separated from the others by simple agglutination. Everyone was surprised to find these Type IV strains a clear-cut, serologically, homogeneous group, with practically no antigenic affinity for Type II. Gordon's original Type IV strains did, indeed, cross-agglutinate with Type II, and antisera prepared from them agglutinated the new American Type IV strains, but the new Type IV strains were not agglutinated by any Type II antiserum.

All Type IV strains recovered during these epidemic years could be traced to a single outbreak in Chicago. None was found elsewhere, either here or abroad, and not more than two or three Type IV strains have been isolated since that time anywhere. So a comparative study of these Chicago Type IV strains with others has not been possible, and we can not yet know whether we have an "American Type IV" which is a "sub-type" of the original Type IV, or whether these Chicago Type IV cultures represent merely one variety or strain that was responsible for that one epidemic. That the latter is a possibility is suggested by our knowledge that meningococcus strains of any type vary a great deal in serological pattern, and that those recovered from a given epidemic may be exactly alike in this respect, especially if the epidemic is an explosive one and the microörganism is passed from person to person rapidly. There is much that is mysterious about the Type IV meningococcus that can not be cleared up until new strains are found. The Chicago Type IV strains comprised approximately 8 per cent of all meningococci recovered and typed by us during the epidemic years of 1928-1930 (11). It was apparently the predominant type of the Chicago outbreak. In Chicago, too, a number of spinal fluids yielded a pigmented member of the genus *Neisseria* which was not a meningococcus. This was named *Neisseria flavescens* by the author (9) who studied 26 strains of it. Epidemiologically this was very interesting. Although other members of the genus *Neisseria* have been found in occasional sporadic cases of meningitis, no such organism had, so far as known, been involved in an epidemic before or since.

Type II strains were also easily separated from other types by simple direct agglutination. There was occasionally some cross-agglutination with Types I and III, but not enough to obscure the true identity, and not more, certainly, than would be expected with antiserum made with old strains. Less than 6 per cent of the spinal fluid strains studied during the 1928-30 epidemics were of Type II. They showed no cross-agglutination with any of the Type IV strains described above. These Type II strains came from widely separated localities and were not identical in sero-



Gradually the conviction grew that although the information obtained about the intricate serological patterns of the meningococci was extremely interesting and might have some important applications, the effort spent in trying to separate Types I and III from each other was not actually profitable from a practical and routine standpoint. It seemed as though a much more satisfactory procedure would be to consider Types I and III together as a broad group, and to designate them as Group I, as had been done previously by Griffith (51, 52), Scott (94), and others (5, 30, 7, 36).

#### EPIDEMIC AND SPORADIC GROUPS OF MENINGOCOCCI

There are kinds of evidence other than serological for considering Types I and III to belong together in Group I. These are epidemiological, clinical, chemical, and immunological.

Records of the typing of meningococci go back to about 1915 in England and to about 1918 in this country. Since then, three great epidemic waves have occurred. The majority of strains isolated and typed during this period fell into the Types I and III, the percentages in this country during the 1928-31 period being 81 and 96, respectively. During interepidemic years the percentage of these fell as low as 6. The percentage of Type II strains, on the other hand, was as low as 4 and 5 during the 1928-31 epidemic period, and as high as 40 to 43 in interepidemic times. Thus we see that Group I (composed of Types I and III) has been responsible for all of the large epidemics of which we have serological information. This conclusion is illustrated in table 3. From the number of reported cases it can be seen that the peak epidemic years have been 1918, 1929, and 1936, with low incidence of meningococcus meningitis in the intervening endemic years. Hedrich (53) has discussed most interestingly this rhythmical recurrence of waves of meningococcus meningitis.

Thus epidemiological evidence indicates that we have an epidemic meningococcus, Group I, and another, Group II, responsible for sporadic cases. Group II meningococci are more common also in non-epidemic carriers; in fact, the percentage of



carriers of this microorganism may sometimes be quite high, and such persons may harbor them for many months or even years.

TABLE 3

*Incidence of meningococcus infection and type distribution of meningococci in the United States since 1915*

YEAR	NUMBER OF STATES REPORTING	NUMBER OF CASES	STRAINS STUDIED	TYPE DISTRIBUTION (IN PER CENT OF THE STRAINS EXAMINED)			
				I and III	II	IV	Other
1915	?	1,403					
1916	?	1,748					
1917	30(?)	4,705					
1918	30(?)	5,749	63	58.6	25.8	2.3	13.3
1919	30(?)	2,417					
1920	30	2,258					
1921	30	2,002	27	31.3	18.7	6.3	43.7
1922	30	1,527	15	6.7	0	13.3	80.0
1923	35	1,506					
1924	35	1,223					
1925	35	1,253					
1926	35	1,700					
1927	38	3,001	4	86.1	5.5	7.6	5.9
1928	40	4,996	57				
1929	46	10,551	89				
1930	44	8,384	82				
1931	41	5,518	26	96.0	4.0		
1932	41	3,102	19	68.0	32.0		
1933	44	2,913	7	57	43	0	0
1934	45	2,500	10	60	40	0	0
1935	43	5,736	91	84.4	13.2	0	2.2
1936	45	6,528	299	89.1	8.7	0	2.7
1937	44	5,484	132	84.0	16.0	0	0
1938	44	2,788	28	53.6	46.4	0	0
1939	49	2,051	33	45.4	54.6	0	0

Nasopharyngeal strains are often so non-specific that it is difficult to group them by agglutination, although they are agglutinated by polyvalent antiserum and have typical cultural and fermentation reactions. During epidemics Group I carriers are more apt to be found than in non-epidemic times, and there is reason to believe that it is usually these Group I carriers that transmit the infection. It is not so much the number of carriers

in a population that is important, as the kind of meningococcus that is being carried. Unless studies on this aspect can be carried through, as by the methods of Rake (91), carrier surveys are worse then useless.

Laybourn (69) has stated his belief that the chronic carriers of Group II strains are responsible for the sporadic cases, intermittently transmitting heavy infections to contacts who might be able to resist small numbers of these strains of lower virulence, but who succumb to the massive doses.

From a clinical standpoint, Group I strains of meningococci act alike. There has been no hint of any difference in clinical manifestation that would indicate the division of this Group into Type I and Type III.

The Group II strains, on the other hand, sometimes tend to produce a clinical picture that is different from the typical epidemic case. Such strains often do not localize in the meninges, but cause a generalized blood-stream infection. These cases often show a rash, which may be more or less purpuric, and they are sometimes mistaken for other infectious diseases, e.g., typhus or Rocky Mountain spotted fever. Although the microorganisms often remain for some time in the blood stream, there are few cases of fatal endocarditis due to Group II meningococcus; fatal meningococcus endocarditis is usually found to be caused by Group I strains.

In many cases in which Group II strains cause a typical meningitis, the illness tends to be prolonged, and sometimes runs a chronic course. Of course, Group I infections may also occur in a septicemic form without meningeal symptoms and with a purpuric rash. But such cases are more apt to be fulminating, and run a quick course.

Chemical studies of the meningococcus have indicated that there is no reason to divide Group I into Types I and III. Rake and Scherp (89) have demonstrated a specific carbohydrate which is common to the two types. This carbohydrate is a sodium salt of a polysaccharide acid (93). They have been unable to demonstrate a comparable carbohydrate in the Group II strains which they have studied.

The close relationship between Types I and III can be demonstrated also by the plate-precipitin, or "halo" method. This was first described by Petrie in 1932 (84), studied further by Kirkbride and Cohen (65), and later developed quantitatively by Pittman, Branham, and Sockrider (85) into a method for making a preliminary evaluation of immune serum. The relationship is also shown by the "quellung" reaction obtained with the Group I strains. This was first demonstrated by Clapp (22). We shall see later that the relationship between Types I and III is also shown by protection experiments.

Group II is much less homogeneous than Group I. The complexity of the group was first noted by Dopter (28, 29), who reported finding  $\alpha$ ,  $\beta$  and  $\gamma$  types of his "Parameningococcus." Many workers since that day have commented upon this heterogeneity. Group II strains seem closely related in agglutination at 56°C., but when other tests are employed differences among strains are very striking. These differences among Group II strains, as well as the contributions made by the "halo" technique and the "quellung" reaction to our knowledge of the meningococcus will be discussed more fully later.

Henceforth in this paper the terms "Group I" and "Group II" will be used to designate the two principal broad groups into which meningococci fall. The term "Type" will be used only where it is intended to designate some subdivision of a group in a special sense.

#### NEW KNOWLEDGE OF THE BIOLOGY OF THE MENINGOCOCCUS

Rake (87) has made a study of the biological properties of freshly isolated meningococci as compared with old stock strains. The older ones showed rough colonies, small and rather dry, whereas those newly isolated gave smooth, moist, glistening colonies. As these smooth colonies were subjected to usual laboratory maintenance, small rough variants gradually appeared and the two forms of colonies occurred side by side on plates. In time, the rough colonies became more and more numerous. The rough forms also became antigenically non-specific and were agglutinated nearly equally by all monovalent

sera. Apparently Group II strains assumed the rough form more readily than those of Group I. Rake found that nasopharyngeal strains were often of the rough colony variety. Smooth colonies are usually 2 to 4 mm. in diameter, but the author has seen them a centimeter or even more.

Two other types of colonies must be mentioned. One is a minute rough colony which appears only occasionally side by side with other kinds, often in fresh cultures. Fishing and replating these colonies has always resulted in the appearance of both rough and smooth colonies, in the hands of the author, and repeated efforts to isolate a minute colony strain proved unsuccessful.

The fourth type of colony noted by Rake (87) and by the author also, is the mucoid type which occurs in a few strains. These colonies may be relatively large, much raised and cushiony. They are very mucoid and stringy and do not emulsify well.

Although he was unable to demonstrate a true capsule by the staining methods which he used, Rake (87) showed that the clear zone so often seen in stained preparations was wider in newly isolated strains than in stock strains, and that the rough variants showed no such zone at all. Since then, Clapp (22) has demonstrated a definite capsule on smooth Group I meningococci by means of the Neufeld quellung-reaction.

Gibbard (45) has shown in his electrophoresis studies that fresh strains have a higher P.D. than stock strains, and this finding has been confirmed by Branham (10).

Rake (88) also investigated the agglutinogenic properties of fresh as compared with stock strains of meningococci, preparing monovalent antisera in rabbits and studying the agglutination obtained with homologous and heterologous antigens. Differences of great importance were found. Sera prepared with fresh, smooth strains agglutinated the homologous antigens specifically and without crossing if the test was incubated for 2 hours at 37°C. and then placed in the icebox over night. When the test was performed with the usual 56°C. incubation over night, crossing was quite pronounced, and results only in the highest dilutions seemed significant. Sera prepared from rough stock

strains did not agglutinate well at 37°C., but gave good agglutination at 56°C. over night. This agglutination was not clear-cut, however, and much crossing occurred.

Apparently specific agglutination occurs first (88), and if smooth specific strains are used for immunization, clear-cut results can be obtained at the lower temperature. If the strains used for immunization are not smooth and specific, specific agglutinins will be lacking in the antisera and little agglutination occurs at 37°C. It is easy to understand how agglutination at 56°C. came to be the usually accepted technique during the years when fresh strains of meningococcus were rarely available. And it is also easy to see that the agglutination titers obtained at this higher temperature and with the overnight incubation time led to a false sense of security about the value of serum to be used therapeutically.

Chemical studies done during this time were enlightening. Rake and Scherp (89, 90), in their studies on the antigenic complex of the meningococcus, separated three fractions. One was a carbohydrate common to all meningococci, and present in some other microorganisms also, which they termed the "C" substance. This is, no doubt, the same carbohydrate which Zozaya (107) had recovered, in less purified form, from other members of the genus *Neisseria* and from certain other microorganisms as well; and it is also related to the alcohol-precipitable carbohydrate found by Miller and Boor (79) in the gonococcus and the meningococcus. It is probably the same as the "C" substance obtained by Tillett and Francis (98) from some of the pneumococci.

Another fraction obtained by Rake and Scherp (90) was protein in nature, and was likewise common to gonococci and to Type III pneumococci. This fraction was very toxic for animals, especially rabbits. Rake and Scherp termed this the "P" fraction.

The third fraction was found to be "Type"-specific and not to be common to all meningococci (89). In Types I and III, this fraction was carbohydrate in nature and identical for the Type I and the Type III strains studied, thus giving chemical evidence for the identity of these two types. Scherp (93) purified the Type I fraction and found it to be a sodium salt of a polysaccharide acid. The Type II specific substance was different in

nature since it gave only a weak Molisch reaction. This substance has been studied recently by Menzel and Rake (76), and they have found it to be protein in nature and antigenic. A "Type"-specific carbohydrate seems to be absent from this group according to these studies.

Recently isolated strains of Type IV have been unavailable for these chemical studies.

Evidence indicates that the specific carbohydrate is responsible for the highly specific precipitation reaction obtained with Group I strains of meningococcus on agar plates containing immune serum. Petrie (84) described the "halos" of specific precipitate which are formed by the interaction of the antibody in the serum with the specific polysaccharide of the meningococcus when colonies of the latter are allowed to grow on agar plates containing the serum. This reaction is not merely species-specific but distinguishes clearly the Group I strains (Type I and Type III) from the Group II strains which do not produce the specific carbohydrate. Petrie found that he could distinguish between rough and smooth colonies in this way. Newly isolated strains of Group I meningococci gave the specific reaction whereas rough, older stock cultures did not. Kirkbride and Cohen (65) confirmed Petrie's observations in a study of many strains. They showed that the length of time during which a culture is maintained in a laboratory is not, in itself, responsible for its loss of specific substance, but rather the conditions under which the culture is kept. Some strains which they have cultured for many years are still smooth. The individual strains vary greatly, too, in their tendency to become rough. The degree of halo production seemed to correlate with the degree of smoothness or roughness, and with the degree of "type-specificity" of the strain as determined by this and other methods.

Maegraith (74) plated out very old Group I strains and found that individual colonies varied greatly in halo production, according to their smoothness or roughness, some giving good halo and others none at all. Pittman, Branham, and Sockrider (85) have utilized this specific halo reaction for preliminary evaluation of therapeutic sera.

Most Group II meningococci have not been found to produce

halos with the majority of polyvalent sera. The significance of this occurrence is not yet clear. It has just been pointed out that no specific carbohydrate has been found in Group II meningococci; nevertheless, some Group II strains do produce halos with sera containing a high content of specific Group II antibodies. It must also be remembered that most polyvalent sera contain very few antibodies that are specific for Group II. If better Group II antisera are produced, it is possible that it will be easier to demonstrate specific halos for Group II strains.

There are some indications that the specific carbohydrate fraction of the meningococcus is associated with the capsular layer of the cell. For one thing, cell washings are peculiarly rich in the specific carbohydrate. Then, too, we know that capsules can be demonstrated by the "quellung reaction" only in smooth strains that produce halos. As a rule, these are Group I strains, although Cohen (23) has reported capsule-swelling in certain Group II strains which we, also, have found to produce halos. In general, Group II strains do not show capsular swelling, nor do they produce halos, although the failure to produce halos may be due to the condition of the experiment.

Little (73) has shown that meningococcus cultures showing capsules behave differently in agglutination from those with no capsules. He referred to capsular agglutinins in the antiserum, and to somatic agglutinins. The capsulated meningococci are agglutinated by the capsular agglutinins and the non-capsulated meningococci by the somatic agglutinins. According to this thesis an antiserum made by immunizing horses with rough, non-capsulated meningococci would contain little or no capsular agglutinin, and the capsulated organisms would not be agglutinated easily by such serum. The non-capsulated antigens were agglutinated better by such sera. Conversely, serum made by immunization with smooth capsulated strains would agglutinate capsulated strains easily and quickly. It is no doubt the presence of the specific or capsular factors that cause specific agglutination; and that is why typing freshly isolated strains is easier than typing the same strains after a period of laboratory maintenance. "Crossing" is due to somatic or non-specific factors, and becomes

more and more pronounced as the strains become less specific. The specific carbohydrate is the same for all Group I meningococci, but the somatic pattern may vary among them greatly. Thus, when rough strains are used, results like those illustrated in table 2 may be found. In typing by agglutination, changing from one strain to another as antigen, or from one serum to another, may result in an apparent change of Type if overnight incubation at 56°C. is used.

Whether we refer to them as smooth, capsulated or type-specific it is plain that meningococci possessing these qualities are very different from the rough, non-capsulated, non-specific forms that all meningococcus cultures tend to become when maintained in the usual routine way. They are different culturally, serologically, and chemically, and we shall see that they also differ markedly in virulence.

#### VIRULENCE OF MENINGOCOCCI

In meningococci, virulence is a transient and unstable quality. So quickly do they lose it after isolation that until very recently the statement was usually made that the "meningococcus is not pathogenic for lower animals with the exception of the monkey." Such large doses of the microorganisms were necessary to kill small laboratory animals that they were considered to have died, in all probability, from the "endotoxins" of the bacterial cells and not from actual infection. Occasionally workers were able to find virulent cultures and to maintain them in this state long enough to do some valuable animal work. Such was the mouse-protection test for antimeningococcus sera that was worked out by Hitchens and Robinson (57). Other workers, lacking virulent cultures, were unable to obtain constant results, since the "endotoxins" in the large doses of their avirulent cultures interfered with their findings. Branham, Lillie and Pabst (13, 16) found many new strains to be virulent enough to produce fatal infection in experimental animals, although relatively large doses of the bacteria were necessary. Rabbits and guinea pigs developed a typical meningitis after intracisternal injection, and in the guinea pig the meningococci were often recovered from the blood.



Mice developed a generalized infection after intraperitoneal inoculation and the meningococcus could be recovered from the heart blood. Within a few days these new cultures of meningococci lost their virulence for these animals and such large inocula were required for killing that death could be attributed to toxicity instead of true infection by the microorganisms. Zdrodowski and Voronine (106), in Russia, also reported successful experimental infection of rabbits with meningococci.

During the winter of 1934-35 there was a sharp increase in the number of meningococcus infections, and many of the strains obtained were of sufficient virulence for a time to infect mice in doses of approximately 100,000 bacteria. Branham (14) used these strains to evaluate antimeningococcus serum by a mouse-protection method. She succeeded in keeping the strains virulent for several months but finally found it necessary to lay them aside for fresher strains. No standardization of method was possible under these conditions.

Two important events occurred about this time which opened the way for rapid progress in knowledge of the meningococcus. One of these was the demonstration by Miller (78) that gastric mucin, as a medium for suspension, brought out, or protected, the virulence of meningococci so that it was possible for as few as 2 to 10 highly virulent meningococci to infect a mouse. This meant that even moderately virulent strains of meningococci could be used for study in animals, and that many multiples of a minimum fatal dose could be used in protection tests without introducing the "endotoxin" factor. Moreover, the meningococci could be kept at maximum virulence for mice by frequent mouse passage, suspended in mucin. By "maximum virulence" is meant that a dose of from 2 to 10 meningococci, in mucin, is sufficient to kill a mouse.

The other important event was the development of the lyophile method of preservation of cultures. Although preservation of cultures by freezing and drying was by no means a new idea, the perfection of the Flosdorf-Mudd (43) apparatus did much to bring this method into an easy laboratory routine. Meningococci live for several years when preserved thus. When culti-

vated again, especially after several rapid transfers, they show the same characteristics that they possessed when they were frozen and dried in vacuum. Thus freshly isolated virulent strains may be kept virulent for a long time.

Newly isolated strains of meningococci vary considerably in their initial degree and duration of virulence for mice. It is much easier to keep some strains virulent than others. Virulent cultures are always smooth, and the Group I strains always produce specific halos and have capsules. The virulence of Group II strains is especially apt to be transient. Fewer Group II strains seem to possess capsules or produce halos, though this may be actually due to the low content of Group II antibodies in serum. Virulent Group II strains are, however, always smooth.

Freshly isolated strains of meningococci differ from each other in many ways besides the duration of their virulence. They vary in the persistence of capsules, halo production, and other evidences of smoothness and specificity. Strains maintained at maximum virulence for mice vary in their susceptibility to phagocytosis. Heist and the Solis-Cohens (54), and Silverthorne (95, 96) have shown that freshly isolated virulent cultures of the meningococcus withstand the lytic action of fresh human blood better than older, avirulent strains. Branham (20) has found this to be generally true, although individual strains, kept at maximum virulence for mice, vary greatly in this respect. She found, too, that they vary in susceptibility to serum- and to drug-therapy (18) when used experimentally in mice. Either these variations are inherent in the individual strains and independent of virulence, or else there is a virulence of a kind not measurable by any of the tests yet applied.

Variation in sensitivity to serum treatment is especially important, since serum-therapy in meningococcus meningitis is so widely employed. Table 4 shows this difference in sensitivity to serum treatment. Six strains of meningococci, each of maximum virulence for mice, were included in a study with one "polyvalent" antimeningococcus serum. It can be seen that the amount of serum necessary to protect 50 per cent of the mice varied from as little as 0.0013 ml. with Strain 1041 (Group I)

to a point where 0.1 ml. gave practically no protection against Strain 1054 (Group II).

Table 5 shows the differences in response of these same 6 strains of meningococci to sulfanilamide (18). All mice were protected from Strains 1041 (I), 1037 (I), and 1054 (II) by the

TABLE 4

*Difference in sensitivity to one antiserum of 6 strains of meningococci, all of maximum virulence for mice*  
(100,000 M.F.D.)

STRAIN	GROUP	AMOUNT SERUM B PROTECTING 50 PER CENT MICE
1027	I	0.0056 ml.
1037	I	0.0015 ml.
1041	I	0.0013 ml.
963	II	0.0065 ml.
1054	II	No protection
1108	II	0.020 ml.

TABLE 5

*Variation in sensitivity of 6 strains of meningococci to sulfanilamide*

STRAIN	GROUP	PERCENTAGE OF DEATHS ACCORDING TO AMOUNT OF SULFANILAMIDE GIVEN				
		1 mgm.	2 mgm.	4 mgm.	8 mgm.	No drug
1027	I	80	10	10	0	100
1041	I	0	0	0	0	100
1037	I	40	0	0	0	90
963	II	60	60	0	0	100
1054	II	60	0	0	0	100
1108	II	80	60	60	60	90

doses used, whereas a small percentage was protected against 1108 (II). Similar results were obtained with sulfapyridine.

It may be mentioned here that the combination of antiserum and drug was far more effective in protecting mice than either agent alone, more even than would be accounted for by the sum of the two effects (15, 18). Apparently a synergistic action is present; and it is not virulence alone that seems responsible for this difference.

When meningococci are established at maximum virulence for mice it is relatively easy to keep them so for indefinite periods by following a routine for their maintenance. Of course, it is simple to dry them in vacuum by the lyophile method and to store them until needed. In this case several rapid transfers (e.g., twice a day) on blood-agar slants should be made before titration of the virulence of the strain (86). But often it is necessary to keep cultures on culture media at maximum virulence over long periods of time. This is especially desirable in the case of those strains used in evaluating sera by a standardized technique. The media that prolong viability for the greatest time are not those that are best for maintaining virulence; indeed, the very opposite seems to be the case as a general rule. Semi-solid agar is excellent for maintaining stock strains for at least a month, but to maintain virulence it is necessary to resort to some other method, such as transfer every two days on blood-agar slants or three times a week on serum-glucose-agar slants. It is likely that the excellent experimental results which Kirkbride and Cohen (65) have reported with old laboratory cultures are due to their having maintained their cultures in a similar fashion. Occasionally mouse passages are necessary to supplement these frequent transfers, these to be done bi-weekly or weekly according to the strain. Cultures grown for 5 or 6 hours on blood-agar slants are best for use when virulence and freedom from autolytic products are desired.

#### MENINGOCOCCI AS ANTIGENS

It is important that something should be said about the behavior of meningococci as antigens in the immunization of animals.

Group I strains are very much better antigens than Group II strains, and smooth strains are much better in this rôle than rough strains. In fact, if the smooth capsular antigens are not present, specific antibodies for these antigens can not be produced and only the rough, or somatic antibodies will be found.

In rabbits, the earliest agglutinins for Group I are the most specific. As immunization continues, the range of the serum-

agglutinins spreads, so that much cross-agglutination between types and groups occurs. Precipitins are much slower to appear than agglutinins.

Rabbits require much longer immunization with Group II strains to yield a serum containing measurable antibodies, whether agglutinins, precipitins, or protective antibodies. In fact, a good agglutinating serum for Group II is difficult to obtain. Precipitins and protective antibodies for Group II are even more unattainable. Individual strains of Group II vary in antigenicity even more than those of Group I. Serum from rabbits injected with one of our standard Group II strains (No. 963) over a period of 9 months showed practically no protection for mice, although the strain was of maximum virulence for mice. Experience with horses has been similar to that with rabbits.

Since Group II strains are generally such poor antigens, it is not surprising that polyvalent therapeutic antimeningococcic serum is usually very low in specific antibodies for Group II strains, and that such sera are often ineffective in Group II infections.

As long as agglutination was the only method of evaluating antisera, these shortcomings of Group II strains as antigens were not obvious. Incubation at 56°C. for 18 hours allows agglutination to occur with rough, somatic, or nonspecific agglutinins and agglutinogens as well as with the specific, smooth capsular ones. A good agglutinin titer at this temperature may thus be sometimes misleading.

Miller's (78) introduction of mucin to the field of meningococcus study has allowed the development of a mouse-protection technique which has been very revealing. This mouse-protection test has not as yet been adopted as the official method of testing antisera for obvious reasons. Every factor involved is variable—mouse, culture, mucin, medium, serum, and individual worker. Pittman has discussed these factors in detail (86). Each must be standardized as accurately as possible so that comparable results can be obtained on various testings and in different hands. After that is done, a trial period will be necessary in order that the results of testing with mice can be correlated or compared with

clinical results. The mouse-protection test has shown that most polyvalent sera have very few specific antibodies for most Group II strains.

The poor antigenicity of Group II strains, in general, and the resulting low content of specific antibodies for Group II in sera may account for the relatively rare demonstration of halos with these strains on serum agar plates. That such halos do occur with some of these strains and good sera has been demonstrated. Since no specific carbohydrate has been demonstrated for Group II, the halo reaction may be dependent on some less active fraction. When better Group II sera are prepared, more light may be shed on this phase of the problem.

It is interesting to note the close correlation that exists between mouse protection and halo production (i.e. the plate precipitin test) in the sera studied. This is especially evident with the polyvalent sera, both whole and concentrated, which are made in various localities. More than a hundred such antisera have been studied (85, 86), using mouse protection, halo production, and agglutination at both 37° and 56°C. The agglutination at 37° is more closely correlated with protection and halo production than is agglutination at 56°. This suggests that it is the specific capsular antibodies that are important in protection. Hence, on an immunological basis there seems to be no reason for separating Group I meningococci into Types. If animals (i.e., rabbits or horses) are immunized with either Type I or Type III strains, the serum from them protects mice against infection with either type. But these sera do not protect against Group II strain infections, nor does a Group II antiserum protect against the Group I (Types I and III) infections.

#### TOXINS OF THE MENINGOCOCCUS

Toxin-production by the meningococcus has been the subject of much discussion. That products of the meningococcus are extremely toxic for animals was recognized in some of the earliest studies on this microorganism. When cultures of low virulence were used in animal studies, the amount of "endotoxins" was often great enough to kill the animals, aside from the infection

induced. Recognizing that these "endotoxins" were the chief lethal factor of such cultures, Flexner (40) used autolysates of the meningococcus instead of living cultures in testing his sera in 1908. This procedure was also recommended by Kraus and Doerr (67). Gordon (49, 50) believed antiendotoxins necessary for good therapeutic sera and described a technique for determining their antiendotoxic potency. He discarded all sera that did not have an appreciable antiendotoxic content as measured by his tests in mice. Results obtained with "endotoxins" were found to be irregular, and this technique of Gordon did not come into general use. For a time the toxic products of the meningococcus were ignored.

In 1931, Ferry, Norton, and Steele (34) described a toxin which they obtained from young broth cultures of certain strains of meningococci. Only those strains which grew as a pellicle at the surface of the medium produced this toxin. After the pellicle had sunk to the bottom of the flask the culture gradually became non-toxic. They considered this to be a true soluble toxin, and produced antitoxin by immunizing horses with filtrates of these cultures. Ferry has (35) reported some neutralization of the toxin with antitoxin in monkeys.

There has been much discussion about this toxin. Some workers have disagreed with Ferry and others as to the nature of this toxin and have insisted that it is endotoxic and the product of autolysis. None of these workers has, however, followed the technique described by Ferry and his co-workers; instead, they have incubated their cultures for a number of days, long enough for abundant autolytic products to accumulate and to mask the action of any weak soluble toxin which might be present.

Laboratory proof of the true toxin nature of this poison is difficult to obtain. The usual laboratory animals are extremely resistant when it is given intravenously, intraperitoneally, or subcutaneously, and so protection with antitoxin according to the law of multiple proportions has not been demonstrable. When it is given intracisternally to rabbits, guinea pigs, or monkeys, very small amounts produce an acute purulent leptomeningitis, often fatal, which is clinically and histologically indistinguishable from

that caused by the meningococcus itself (13, 16). Since horse-serum is itself very toxic for the meninges of rabbits and guinea pigs, neutralization with antitoxin injected by the intracisternal route has not been demonstrated (17).

At present toxin is demonstrated by intradermal injection into human subjects (34). Approximately 50 per cent of individuals give a positive skin test with Ferry's toxin in high dilutions. The antitoxin is standardized by the partial neutralization of a positive skin test made with the toxin (34). This is admittedly unsatisfactory, but it is the only method of toxin-antitoxin neutralization that is now available.

It is evident that filtrates of certain strains of meningococci, grown in a special broth medium for 24 to 48 hours, contain a substance or substances highly toxic for some laboratory animals when given intracisternally, producing positive skin reactions in a high proportion of human beings. Some strains are apparently much better than others for this purpose. This toxin is produced only by cultures growing at the surface and forming a pellicle, and it seems to disappear on prolonged incubation. Later, much "endotoxin" accumulates. Although it is possible that the toxin is very dilute "endotoxin," as is claimed by some, it is also quite likely that these two toxic products are distinct from each other. Whatever the nature of this product may be, those who have followed Ferry's technique have found this toxin, although they may not have found it for all of the serological types.

Antitoxin produced by the immunization of horses with Ferry's toxin is very low in content of agglutinins, precipitins, and antibodies that protect mice against infection, but many clinicians feel much enthusiasm for it as a therapeutic agent. The discovery of some satisfactory way of demonstrating toxin-antitoxin neutralization for the meningococcus will be a welcome event.

#### PRESENT CLASSIFICATION OF MENINGOCOCCI

By way of a summary, and at the risk of perhaps needless repetition, we shall conclude the discussion with the following remarks.

In some ways the classification of meningococci has become



simplified. On epidemiological, clinical, chemical, and immunological grounds there seems to be no real reason for dividing Group I meningococci into Types I and III. This division can be done serologically with the majority of the strains of this group by absorption of agglutinins if enough time and effort are spent, and if the strains and sera used as a basis of the study are sufficiently specific. But the interrelationship of these two types is so close that a change of standard strains can result in a change of the resultant typing.

Epidemiologically, there are found only two main groups of meningococci, Group I and Group II—an epidemic group and an endemic group. These differ from each other in clinical manifestation, chemical composition, and immunological behavior. The endemic Group II includes most carrier strains, and a high proportion of those from sporadic cases. Group I has been responsible for most of the epidemics during the past 25 years, i.e. the period over which we have serological information.

Group II is more heterologous than Group I. This was recognized as early as 1914 by Dopter and Pauron (28, 29), who described  $\alpha$ ,  $\beta$ , and  $\gamma$  types of their "Parameningococcus." Representative cultures of these types are no longer available, so we have no way of knowing whether or not they are related to some of the peculiar Group II strains that are encountered. All of the latter strains which are agglutinated by Group II antiserum are considered to fall into Group II, though variations among them in serological pattern may, at times, be very striking. Other differences may be found among these Group II strains. Only a few of them have been found to have capsules as demonstrated by the quellung-reaction (23). These have invariably been found to produce conspicuous halos with polyvalent sera that yield no halos with other virulent, smooth Group II strains (19). Immunologically, these strains seem quite different from the other Group II strains. They are good antigens, and serum from rabbits immunized with them protect mice against the homologous and related strains, but not against the other Group II strains (19). Conversely, sera which protect against the other Group II meningococci do not protect against these capsulated

strains. There has been no chemical work reported upon this subgroup, so that at present we have no knowledge as to whether or not there may be a capsular carbohydrate specific for it. Possibly these strains may not belong to Group II at all, in spite of their agglutinogenic affinities.

Menzel (77) has worked with certain Group II strains which are serologically comparable to those used as "standard" Group II strains for typing and serum-testing. He has obtained no carbohydrate there, but has found the specific fraction to be protein in nature. Since the strains with which he has worked are typical of the usual Group II strains, he and Rake are referring to these strains as "Type" II and are considering them as a type within Group II. The capsulated halo-producing Group II strains referred to above show important differences from these.

Except for those recovered during an epidemic, nasopharyngeal strains are usually of Group II. These are frequently rough and non-specific, whereas those from spinal fluid and blood are usually smooth and specific. Some nasopharyngeal strains are so completely rough that they can not be typed. These are sometimes agglutinated equally well by both Group I and Group II antisera. Since they are probably devoid of the specific fractions, even absorption of agglutinins would not give satisfactory results.

The "Type IV" strains that have been found in this country have not had the close relation to the "Type II" strains of Gordon and Murray that their original strains had. Type IV strains are very rare in the United States now and those which are maintained here are too old for their relationships to be accurately determined. Possibly they, too, represent a subgroup or type within Group II; or they may be an independent group.

We may summarize the subject of classification of meningococci by saying that they fall into two broad serological Groups, I and II. These may be divided into subgroups or Types. The Types I and III of which Group I is composed, are alike epidemiologically, clinically, chemically and immunologically, so that there seems to be no reason for separating them as a routine procedure. Group II is apparently also composed of subgroups or types.

Immunological and probably chemical differences within this group are becoming more apparent since the development of the mouse-protection and other techniques. There seems to be at least one subgroup that is immunologically distinct, and it is possible that the separation of this subgroup may prove to be of practical clinical importance.

The relation of the "Type IV" of Gordon and Murray to the present Groups I and II is not understood.

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# NUTRITION OF THE DIPHTHERIA BACILLUS

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Concerning the earliest reported attempts toward the cultivation of the diphtheria bacillus in media free from protein or its higher cleavage products, little need be said. That they were carried out indicates a recognition of the probable importance of the ability to grow the organism in media of known and simple composition. That they should have resulted in practically complete failure was, in the light of our present knowledge, inevitable. Protein chemistry was still in its infancy. Certain of the amino acids were still unknown. The conception of accessory growth substances, or vitamins, had not yet been introduced, although it is noteworthy that this was to come within a relatively short time through studies with another unicellular organism, yeast (Wildiers' "bios," 1901), more than a decade before it was encountered in the study of animal nutrition.

Consequently the experiments of Guinochet (1892) with "proteid-free" urine, and of Uschinsky (1893) with a medium containing inorganic salts, sodium aspartate, and glycerol were foredoomed to failure. The latter, it is true, claimed to have obtained some growth, but without details of his experiments, the purity of his materials, the size and nature of inocula used, or quantitative data on the amount of growth observed, one may safely assume that it must have been relatively feeble. Attempts to repeat his observations on the part of Fraenkel (1894) and of Hugounenq and Doyon (1896) met with complete failure. To Uschinsky, however, appears to go the credit for being the first to attempt the cultivation of this, and other pathogenic organisms, by means of a reasonably constituted inorganic salt mixture together with chemically defined sources of nitrogen and carbon.

Later, Hadley (1907 a and b) undertook the repetition and extension of Uschinsky's early experiments with the diphtheria bacillus, introducing the amino acid glycine into the medium as a source of nitrogen. A measure of success was reported with some strains, failure with others. One may, perhaps, speculate as to the nature of some of the organisms which grew, described by Hadley as tremendous bacilli of more than 10 micra in length, forming so tough a pellicle that the tube could be inverted without causing its rupture. This modification in morphology and nature of growth he ascribed to alteration accompanying adaptation to the medium. It does not appear to have been observed by later investigators.

Toxin formation, in these early experiments, was demonstrated by the injection of varying amounts, from 0.5 ml. to 1.5 ml. or more of *culture* into a guinea pig, and, as far as one can make out, without the use of control animals protected with antitoxin.

For nearly ten years after the appearance of Hadley's papers very little seems to have been attempted toward an advancement of the general situation. From about 1917, when contributions on the subject began to appear from Rettger's laboratory (Robinson and Rettger, 1917) and continuing to the present time, a considerable volume of material has been accumulating. In order to review this in any detail, certain general considerations regarding objectives and directions of approach are essential, and the implications following from variation in strains must be indicated.

#### OBJECTIVES

It is essential to direct attention to this subject because there are at least two different criteria of growth used by the various contributors to the literature. From the following considerations these may be differentiated by the terms "detectable growth" and "normal growth."

If one implants a living bacterial cell in a tube of sterilized medium, and holds the culture at a suitable temperature, one of three things must necessarily occur: either the cell will (a) die, (b) remain more or less permanently dormant, or (c) it will

multiply. If several or many cells are used the situation becomes more complicated, because the death and disintegration of some of the cells may so alter the composition of the medium that some of the survivors begin to multiply. In either case, cell division, if it occurs, may proceed in a variety of ways. The ultimate result will depend on the rate at which division takes place, and on the number of successive generations produced. These facts are all perfectly obvious and have been extensively dealt with by students of cell physiology.

"Growth" may be presumed to have taken place with the division of a single cell into two during an infinite time interval, but its detection at this level would be impracticable. "Detectable growth" begins at some arbitrary point which will depend upon the method used for its determination, but which for practical purposes must imply something quantitatively more definite than delayed fission of a single cell. It will, on the other hand, be far short of the growth which the cell in question is known to be able to attain under optimal conditions. For example, if one represents graphically the degree of growth by a line extending from a zero base to a maximum of 100, detectable growth would fall at any point between perhaps 1 and 10, depending on the method chosen and accuracy of observation.

The significance of these comments, which may appear entirely academic, lies in the fact that the choice of one level of growth, rather than another, is governed by the purpose of the individual investigator. "Detectable growth" has been used by Braun, from whose laboratory have come a number of widely-quoted contributions dealing with the growth of the diphtheria bacillus, to determine the simplest requirements compatible with cell division. He states (Braun, 1931) "Es kommt nicht auf Üppigkeit und Schnelligkeit des Wachstums, sondern nur auf die Beantwortung der Frage der Unentbehrlichkeit und der unmittelbaren Assimilierbarkeit der dargebotenen Nährstoffe an." This, of course, represents a perfectly justifiable objective, and has resulted in valuable observations on minimal nutritive requirements. Yet even Braun in describing his experiments, introduces a kind of quantitative measure of growth together with a time

element when, for example, he states (Braun and Mündel, 1929) that in a particular medium a strain of the diphtheria bacillus grew "rapidly and vigorously," so that five passages could be obtained in 24 days, while a reduction in concentration of one of the components, sodium aspartate, to one tenth, often resulted in much less vigorous growth, 91 days being required for five passages.

The term "normal" as applied to multiplication of bacteria also requires further explanation. The quantitative nature of the phenomenon is inherent in the conception. There must be a standard of comparison, and the experimental results must be stated in some more or less reproducible units, whether they be symbols, (+ to + + + +), turbidity measurements, diameter of colonies in millimeters, or weight of bacterial substance in milligrams. The more definite and reproducible the units, and the further removed from individual errors in judgment, the more satisfactory the results are likely to be. As a basis for comparison, it seems reasonable to use the amount and type of growth commonly manifested by any particular microorganism on the usual empirical medium ordinarily used in its cultivation. Such media are the outgrowth of years of observation, and, as a rule, represent at least a fairly satisfactory degree of multiplication. If, in the course of an investigation, it is found possible markedly to increase the amount of growth obtainable in empirical media, by the omission of inert materials, and by suitable increases in concentration of essential factors, such growth is not necessarily abnormally great, and a readjustment of what is held to be normal for that particular organism must be made.

The purpose for which normal growth has been taken as an objective is fundamentally practical. It is an attempt to obtain a reproducible medium of known composition to replace the empirical and complex mixtures now in general use. An adequate appraisal of results must therefore take all these facts into consideration.

Finally, a word must be said regarding differences between growth of an organism in first transplant to a simple medium and its ability to maintain itself through repeated subculture on such

a solution. In this connection one must consider the possibility of transfer of metabolites with the inoculum which may quickly disappear through dilution in subsequent transplants. To offset this, the well-known ability of microorganisms to adapt themselves to an unfavorable environment, depending on the acquirement of new synthetic properties, must be taken into account. An attempt to reduce to a minimum the possibility of transfer of metabolites may add considerably to the complexities of an already difficult situation, whereas the modification of requirements through adaptation may lead to false conclusions. The procedure which is selected and the results obtained must be considered in the light of these facts.

It has long been known that *Corynebacterium diphtheriae* grew well and formed toxin on the usual nutritive bouillon. Blood serum was believed to favor its growth (Loeffler's medium), and a host of complex materials, milk, unheated organ emulsions, and the like had been suggested at one time and another as exerting a favorable influence. In spite of these facts, the work of most investigators on growth requirements of the diphtheria bacillus has been planned along what may be called "synthetic" lines. To the usual salt mixtures were added various known and more or less possible sources of nitrogen and carbon in various combinations and permutations. Incubation was continued for days, or even weeks, apparently in complete disregard of the fact that the organism grows well in 18 to 24 hours on a suitable medium. Had the growth requirements of the organism proved to be simple, such methods would undoubtedly have been the most rapid and direct. Complicated as the situation proved to be, they could have been completely successful only by the most improbable accident. Even so, as will be seen, certain facts of perfectly definite nature did emerge from such work.

Quite the opposite method of approach, which may perhaps be spoken of as "analytical" in character, would begin at the other end. An empirical medium suitable for normal growth, as the bacteriologist knows it, could be broken up into its components, and an attempt made to identify the latter individually, either by direct isolation or by substitution of the known compounds most

likely to be present in a particular fraction. Such a method is almost certain to be slow and laborious, but should be successful eventually, and should result in a medium which equals or exceeds the original empirical one, since each component can be adjusted to the concentration leading to maximal growth.

In effect, the synthetic approach assumes that all the chemical factors essential to the normal development of a microorganism are substances already known and of recognized physiological importance. The analytical method makes no such limiting assumption, and it has become increasingly evident that the compounds of nutritional importance, in bacteriology as in animal physiology, include a considerable number which could not be anticipated and supplied by a method of trial and error. Some of these, in the case of the diphtheria group as in other groups of microorganisms, have now been singled out and identified. Others remain obscure. On the whole, recent progress has been unexpectedly rapid, and one may predict that this will continue in the immediate future.

#### DIFFERENCES BETWEEN STRAINS

Before considering in detail the nature of the specific materials which have been shown to be required for the growth of *C. diphtheriae*, it is necessary to discuss the matter of variation between strains. These differences have long been recognized, the partial successes reported by Uschinsky and by Hadley being claimed only for certain strains. Braun and Hofmeier (1927) were probably the first to call attention to this phenomenon when they stated that certain strains of the organism were "anspruchsvoll" or exacting, while others, growing more readily, were non-exacting or "anspruchslös." Successful cultivation on extremely simple media resulted only when representatives of the latter type of strains were used.

The reviewer is unable to write from personal experience with the non-exacting strains, since it has been impossible to obtain cultures of such organisms from Dr. Braun. It is difficult, therefore, to appraise his results on a basis at all comparable with those known at first hand. Significantly, however, in presenting his successful experiments, Braun states that considerable periods

of time were required in order to pass these strains through several generations, (see above). There is no way to judge how well these cultures grew, as compared, for example, to the growth of the same strains on bouillon. Evidently no pellicle-formation occurred, and the extent of growth was estimated by observing the turbidity after shaking. During such long periods one must assume that some adaptation has taken place on the part of the bacterial cell to its environment, and that there need be no direct relationship between the composition of the medium and the original nutritional needs of the microorganism.

There can be no doubt whatever that wide differences in nutritional requirements do exist within the group of organisms classed together as *C. diphtheriae*. It is, therefore, altogether probable that strains of much less exacting nature than any of those which the writer has encountered have been the subject of investigation by others. Similar differences are found among strains of all species of pathogenic bacteria that have been studied, and are probably explicable along lines of functional loss through disuse and re-adaptation. This theory, which attempts to account for differences in growth requirements among bacteria, has been clearly enunciated by Knight (1936). Briefly, it states that the autotrophic bacteria which are capable of growth on the simplest types of inorganic salts, ammonia or nitrate and carbonate, are fully equipped with synthetic mechanisms for the complete production of all of the elaborate chemical groupings of protoplasm. These are held to be the primitive members of the race, since they would have been capable of existence in the absence of complex animal or vegetable materials. In an environment providing an increasingly abundant supply of organic material, certain bacteria, finding many of the components for their own protoplasm already formed, gradually began habitually to depend on such sources for certain groups of substances. Finding it no longer necessary to provide a mechanism for the building up of these particular chemicals, the native synthetic ability for them was gradually lost. Such organisms, placed artificially in a simpler environment, would now have to be supplied with the substances which they had lost the ability to produce.

There are two consequences of such a state of affairs which are



open to experimental verification. In the first place, the adapted cell should still possess the inherent synthetic ability of the primitive one, and it should be possible to train it to regain the lost function. In the second place, failure to require a specific grouping by a given culture should imply that it is able itself to produce the compound in question. Thus, for example, strains of bacteria which do not require tryptophane in their culture medium should be able to synthesize it from simpler compounds. Both of these probabilities have received experimental support which need not be detailed here (see Knight, 1936). For the present, then, this general theory is of considerable service in helping toward an understanding of the descriptive facts which will follow.

We are now in a position to make a general statement of the framework upon which the facts already known in connection with the nutritional requirements of diphtheria bacilli may be assembled. It should then be possible to indicate where further work in collecting new facts is necessary in order to complete the structure. One must balance the possible value to be derived from the filling in of one gap or another in our knowledge, against the time and experimental difficulties likely to be encountered in doing so.

So far as is known, bacterial protoplasm in general, and that of *C. diphtheriae* specifically differs in no fundamental chemical particular from the protoplasm of cells of higher orders of life. Protein, made up of the usual assortment of amino acids is encountered here as in other cells. Nucleic acid, certain carbohydrates and lipoidal materials are characteristic of it as of living material in general. Organic sulfur is present, as well as phosphorus compounds and the ordinary mineral elements. Whichever of these essential building stones the organism cannot produce for itself must be supplied in the medium, and such a compound or element becomes an essential factor for the growth of the organism. If a particular grouping can be built up by the cell only slowly, it may become the limiting factor in growth,

and while not being strictly essential, growth may be greatly hastened or improved by its presence.

We may use, as our criterion of normal growth, that which occurs on some well-known empirical medium in general use. To say that such growth is "normal" is not necessarily correct, but it offers at least a rough standard which will be familiar to every bacteriologist interested in the field. If the growth on a solution of known composition is slower or less in amount than on bouillon, in the case of the diphtheria bacillus, it therefore implies either a deficiency of one or more factors to be found in the bouillon, a badly proportioned formula, or the presence of some inhibiting material.

It is obvious that chemical substances which will supply all the essential elements must be used. Hence nitrogen and carbon compounds, sulfur and phosphorus in some form, and Na, K, Mg, Ca, and Cl, and possibly traces of other elements have to be provided. In addition, certain organic compounds containing specific atom groupings,—the growth accessory substances,—are likely to be required. We shall therefore review the state of our present knowledge of the nutrition of the diphtheria group under the heads of "Nitrogen sources," "Carbon or energy sources," "Sulfur," "Mineral requirements," and "Accessories."

Since so much of the work done with this group of organisms has been inspired by the hope of obtaining a better toxin, or at least one comparatively free from complex substances other than products of bacterial growth, it is desirable to give some consideration to the phenomenon of toxin production, but to attempt a complete résumé of the vast amount of work on this matter would be quite beyond the scope of the present review. The situation in regard to diphtherial toxin will therefore be summarized only to the extent that more precise experiments in the nutritional field have seemed to contribute to the better understanding and control of its production.

#### NITROGEN SOURCES

The traditional media are Loeffler's coagulated serum and peptone-meat infusion bouillon. Since the former is prepared by

adding serum to the latter, it is evident that the possible sources of nitrogen occurring in the usual empirical media which are capable of bringing about "normal" growth are extremely numerous. They include in addition to all the amino acids, peptides of varying degrees of complexity, up to fragments of protein just below the stage of being heat-coagulable, and of the most varied composition. Another large group of muscle extractives, many of which may be assumed to be but imperfectly known, have also to be considered. It is our immediate concern to determine which of these nitrogenous materials can serve the diphtheria group as foodstuffs from which its specific protoplasm may be constructed.

It seems to the reviewer to be a great pity that the rather vague chemical concept of "peptone" has for so long a time been held in apparent veneration by many bacteriologists. Initiated, perhaps, by the observations that certain "peptones" were better than others for the growth of this or that organism, or that some particular brand appeared to yield a better grade of toxin, the illusion has been fostered through commercialization. By directing attention to the large mysterious proteoses, the lowly but useful amino acids and accessories have been kept effectively out of sight. It is even possible that the promoters of the products have deluded themselves. If the specificity of a protein depends, however, as all the evidence indicates, on the arrangement of certain amino acids in a particular order, then it is indeed difficult to see how a huge fragment of a molecule of ox fibrin or of casein can be fitted in to the building up of a molecule of diphtheria protein. We do not, of course, know the methods by which new protein molecules are built up, but it is reasonable to suppose that it must occur by single units, or at most by groups of two or three which happen to suit the needs of the particular instant. In support of this view are the experiments of Berman and Rettger (1918) indicating no utilization by the diphtheria bacillus of purified proteose.

It is possible that instances may be encountered in which larger fragments of the protein molecule may serve as metabolites, but so far as our present information is concerned, such a state of

affairs would have to be experimentally established, and cannot be taken for granted.

Reasoning as above, one is justified in a mental simplification of the problem by eliminating all complex materials from consideration, at least provisionally, after which there remain only amino acids and simpler extractives as the most probable sources of nitrogen in the usual media. How far the process of elimination and simplification can extend, becomes now a matter for experiment.

The earliest attempts to substitute ammonia, aspartic acid, or glycine ended in what must be considered as failure. More complex mixtures, including glycine, leucine, tyrosine, aspartic acid, arginine, and lysine, failed in the hands of Galimard and Lacomme (1907) and similar failures have been reported by Koser and Rettger (1919) and by others.

In 1918 Robinson and Rettger reported successful cultivation of *C. diphtheriae* on hydrochloric acid hydrolysates of casein, lactalbumin and edestin. Their materials were so prepared as to make fairly complete breakdown of the proteins to the amino acid stage seem probable. The excess HCl was removed by evaporation on the water-bath. The resulting growth on media prepared with these materials was definite, but not good. It was greatly improved by the addition of Liebig's meat extract, which of itself, gave very poor growth. It is unfortunate that this work, which appears to have provided a definite basis for successful continuation, should not have been followed up.

A year later Davis and Ferry (1919) attempted to build up a synthetic medium from various amino acids and inorganic salts, together with glucose and certain muscle extractives such as creatine, creatinine, xanthine and hypoxanthine. Growth failed unless a very small amount of meat infusion was present. Here again was a clue which could profitably have been followed. It is noteworthy that this work of Davis and Ferry showed definitely that the amino acids cystine and tryptophane were concerned in the nutrition of their strain (a Park No. 8). Curiously, they appeared to act interchangeably on growth, and, moreover, both glutamic and aspartic acids showed similar effects. Sulfuric

acid hydrolysates of gelatine and gliadin were also used, but with no great measure of success.

Parenthetically, the reviewer wishes to admit a certain confusion in his mind over the term "sodium asparaginate." Uschinsky (1893) used "natrium asparaginicum." Fraenkel (1894) used "Asparaginsäures Natrium." Presumably, each of these is sodium aspartate. The amide of aspartic acid, asparagine, is often incorrectly stated to have been used by Uschinsky, and perhaps for this reason has often been used by other bacteriologists. Whether the English use of the term sodium asparaginate has always been employed to denote sodium aspartate, or whether a solution of asparagine in NaOH may have been used, is not clear. Probably the results would differ in no great particular, except that there is evidence of a very specific nutritional effect of glutamine as opposed to glutamic acid (McIlwain et al., 1938),—a perfectly parallel situation.

Braun and Hofmeier (1927) reported that most freshly isolated diphtheria bacilli can be cultivated on a synthetic medium of the following composition:

	<i>gram</i>
Na <sub>2</sub> SO <sub>4</sub> .....	0.5
MgSO <sub>4</sub> .....	0.005
KH <sub>2</sub> PO <sub>4</sub> .....	0.05
K <sub>2</sub> HPO <sub>4</sub> .....	0.15
Na aspartate.....	0.5
Cystine.....	0.0125
NaC <sub>2</sub> H <sub>3</sub> O <sub>2</sub> .....	0.5
H <sub>2</sub> O to 100 ml.	

They state that cystine is indispensable, whereas the aspartic acid can be replaced by glutamic acid. Later Braun and Mündel (1929) reported the possibility of still further simplification of the medium by the omission of salts of K, Mg, Cl and SO<sub>4</sub>, obtaining successful growth under these conditions. Extending the work, Braun, Hofmeier and Mündel (1929) showed that certain other amino acids could not replace the aspartic acid in the medium. They also showed that strains carried for many passages on the medium retained virulence for the guinea pig and even produced toxin in the medium. A toluol-killed culture was

centrifuged, and 5.0 ml. of the clarified fluid injected subcutaneously into a guinea pig. Considerable swelling and edema appeared, followed by necrosis, but the animal survived. A control animal protected with antitoxin suffered no injury from a similar injection of the material.

Maver (1930, 1931 a, b) has reported confirmation and some extension of Braun's work. She states that strains became adapted to the medium, in which the cystine content was increased somewhat over that of the original formula, and glycine was added. Toxin with an M.L.D. of 0.1 ml. was produced by one strain, while one of Park No. 8 gave a weaker toxin, 0.5 ml. being required to cause death in a guinea pig. Nitsch (1933) has reported a somewhat similar confirmation of Braun's observations.

It must be recalled that Braun has claimed success with his medium only for the non-exacting strains of the diphtheria bacillus. Others failed completely to proliferate. This latter group includes his strain of Park No. 8, and a certain number of freshly isolated organisms. It would be interesting to know something further of the fundamental differences between these two categories in terms of more definite growth requirements.

Mueller and his collaborators (1933) have approached the matter from the point of view of obtaining "normal growth," and proceeded by what has been referred to as the "analytical" method. They also employed a quantitative method (Mueller, 1935 a) of estimating growth by means of nitrogen determinations on the centrifuged and washed bacteria, which was laborious but extremely useful.

The usual nutrient bouillon was considered to be composed of commercial peptone and meat extract. An acid hydrolysate of casein together with tryptophane, which is for the most part destroyed by acid, adequately replaced the peptone (Mueller, Klise, Porter and Graybiel, 1933). By a process of progressive separation of the amino acids of the hydrolysate into fractions, and substitution of known amino acids, so far as possible of synthetic origin in order to avoid the addition of unexpected physiological substances as impurities, it was possible in the case of the

strain investigated eventually to replace the hydrolysate completely by eight amino acids (Mueller, 1935 b, c). These were glycine, valine, phenylalanine, glutamic acid, methionine, histidine, cystine and tryptophane. A strain of the Park No. 8 organism was then investigated in the same manner by Mueller and Kapnick (1935) and again, with a somewhat different assortment of amino acids, including valine, leucine, methionine, cystine and glutamic acid, as good growth was obtained as in ordinary bouillon. Liebig's extract and inorganic salts by themselves giving minimal amounts of growth were used in the preparation of all such media. The presence of certain amino acids in the meat extract in sufficient quantity to supply the needs of the organism was not excluded, so that conceivably both lists may be longer.

Because of certain striking differences in the apparent amino acid requirements of two strains, other cultures were investigated, including "Park No. 8" organisms from a number of different laboratories. It soon became evident that considerable differences existed, even between the various Park No. 8 strains, and that little profit was likely to result from an indefinite extension of that line of attack.

Considerably later, when it had become possible to substitute known compounds for Liebig's meat extract, the amino acid requirements of a third strain were investigated (Mueller, 1938). Here it was found that valine, proline, aspartic and glutamic acids, cystine, methionine and possibly tyrosine had to be supplied, and even with these, growth was never more than about 85 to 90 per cent as good as in the presence of a complete acid hydrolysate of casein. The question was left open as to whether this may have been due to the presence of some unknown material in the hydrolysate, or simply to the fact that amino acids not supplied had to be synthesized and that as a result there was a certain loss in growth efficiency.

The common factors which developed in regard to amino acid requirements of the various strains, (both published and unpublished results), may be summarized in this way: Either glutamic or aspartic acid seems to be utilizable in greatest absolute amount,

evidently to serve as the source of N and C linkages for the synthesis of amino acids and other compounds not supplied in the medium. With some strains these compounds are quite interchangeable, while with others, one or the other is distinctly better. It is possible in the case of both amino acids to use synthetic materials, thus ruling out the possibility of the presence of active impurities. Only the naturally occurring isomer is utilized in each case. Asparagine appears to be quite as satisfactory as aspartic acid. This supplies corroboration of Braun's observations, and of the value of the quite general use of aspartic acid in much of the earlier work. It also supports the conclusion of Abt (1925), who furnished evidence of quite a different sort that glutamic acid was utilized in considerable quantities.

Of the other amino acids, cystine, or some other unoxidized sulfur compound (see section on sulfur), is essential to all strains. Methionine does not replace cystine, but represents for all strains an additional requirement for *maximal growth*. Tryptophane is definitely required by certain strains, not at all by others. In the case of one or two, there is evidence of improvement of growth by minute traces, and inhibition by larger amounts. One or more of the simple monoamino, monocarboxylic acids (glycine, valine, or leucine) are required for optimal growth by all strains. The growth finally attained on such solutions is several times that given by ordinary bouillon, since it is possible by means of the quantitative method employed to push to the optimum the concentration of each factor used. The organisms grow in the form of a typical pellicle which forms in about 12 hours and thickens progressively up to about 72 hours.

#### CARBON SOURCES

Although, carbon is obtained by the diphtheria bacillus from the amino acids which it assimilates, much more abundant growth takes place in the presence of non-nitrogenous organic compounds belonging to the general groups of carbohydrates, acids and alcohols. It will not be our purpose to consider the mechanism by which these substances are utilized, but merely to outline the nature of the materials, and consider briefly the end-products



and the effect on the growing culture brought about in this phase of its metabolism.

From the earliest experiences with the diphtheria bacillus in broth culture, it has been recognized that an initial acidity was likely to develop, which if it did not become too marked, eventually gave place to a slowly developing, alkalinity. In general, the acidity was attributed to products arising from the breakdown of glucose, or some other fermentable material. The later alkalinity was variously held to be due to the formation of ammonia, organic amines, carbonates, etc. The phenomenon has been investigated by numerous workers, particularly in relation to toxin-formation. It has become a part of the tradition attending this matter that an initial acidity must appear, which must be followed by a "reversal" of the reaction, with the development of alkalinity, and that toxin is formed only under these conditions. Among producers of diphtheria toxin have been those who have insisted that sugar-free broth must be used, while others have emphasized the benefit to be derived from the addition of sugars, both glucose and maltose, to the medium.

There is no doubt that glucose is rapidly fermented with acid-formation. With maltose this is not the case, yet the addition of this sugar to the medium greatly improves the growth. Sugg, Fleming and Neill (1927) have shown the presence in the organisms of a heat-labile maltase which breaks the maltose down to the hexose stage. This hydrolysis proceeds slowly (Tasman and Brandwijk 1936), so that the glucose is fermented and its fermentation products are further oxidized as rapidly as they are formed (see below). Maltose, therefore, serves as an ideal source of energy for the growth of the cultures.

The authors just quoted (Tasman and Brandwijk, 1938) later presented evidence that the fermentation of the glucose molecule by the diphtheria bacillus resulted in the appearance of formic, acetic, propionic, lactic, and succinic acids, and ethyl alcohol. Braun and Hofmeier (1927) showed that acetic, lactic, malic and succinic acids, as well as glucose, could be utilized in their very simple medium. Glycerol has been used (Uschinsky, 1893; Hadley, 1907) from the time of the earliest work, and was shown

by Schmidt (1933) to be utilizable, using Braun's method. Formic, propionic, oxalic and tartaric acids are not utilizable according to Braun, Hofmeier and Mündel (1929). The slight discrepancies are easily explained as due either to differences in method, or to variation among strains. That this occurs has been observed by the writer in differences in the effects exerted by glycerol, *d*-lactic acid, and ethyl alcohol on several strains of the Klebs-Loeffler bacillus.

Abt (1925) and Abt and Loiseau (1925) have carried out studies on oxygen-consumption and CO<sub>2</sub>-production by this organism. They have shown that the gas exchange is considerable, amounting to as much as 4 grams CO<sub>2</sub> per 1100 ml. of medium, with a corresponding utilization of O<sub>2</sub>. From unpublished experiments, the writer is inclined to believe that these figures fall well short of the quantities involved when maltose is present in the medium, since he observed the utilization of approximately 200 ml. of O<sub>2</sub> by 30 ml. of culture. Abt believes that many of the amino acids are deaminized, and that the resulting fatty acids are largely burned to CO<sub>2</sub> and water. This, he indicates, is particularly true of glutamic acid.

It appears that the above chain of events offers a perfectly adequate explanation of the series of alterations in reaction in the growing diphtheria broth culture. If hexoses are present, an initial acidity develops, due to the rapid (anaerobic) fermentation to organic acids. In their absence, some acidity may still develop due to changes in certain amino acids. If too much glucose is present, probably more than about 0.2 per cent (Tasman and Brandwijk, 1936), the degree of acidity attained is such as to check further growth of the organisms. With small amounts, the organic acids formed are oxidized to CO<sub>2</sub> and water with a gradual return of the pH to its original level. A further increase in pH results from changes in the amino acids with the formation of NH<sub>3</sub> or other basic substances. Obviously, if sodium acetate or sodium lactate is added to the medium, the oxidation of the organic acid leads to the formation of sodium carbonate or bicarbonate, the initial acid phase is slight or absent, and a more marked alkaline reaction is soon reached. One could

probably control the course of the pH changes throughout the period of growth by the use of suitably chosen quantities of glycerol, ethyl alcohol, sodium lactate, glucose and maltose, together with some regulation of the oxygen supply and CO<sub>2</sub> removal.

#### SULFUR SOURCES

There is general agreement in the literature that sulfur in the form of cystine is acceptable to all strains of the diphtheria bacillus which have been investigated. Reference has already been made to the work of Davis and Ferry (1919), who showed that the addition of this substance to plain meat infusion, itself just capable of maintaining growth, rendered it capable of producing heavy vegetation.

Hosoya and Kuroya (1923) reported positive results when cystine was used together with other amino acids and an inorganic salt mixture. Braun and Hofmeier (1927) found it to be essential in the growth of their non-exacting strains. Similar findings are reported by Gibbs and Rettger (1927), Maver (1930) (1931 a), Hosoya, Ozarva and Tanaka (1933), Nitsch (1933), Schmidt (1933) and others. Braun and Mündel (1927) have suggested a practical application of the observation. They find that the addition of cystine to ordinary Loeffler's medium greatly improves the growth of *C. diphtheriae* on it, without affecting that of other organisms.

The earliest reported synthetic media, such as Uschinsky's, contained inorganic sulfate, as did some of Braun's media. Braun and Mündel (1929) later obtained evidence indicating that this was not essential, and so far as can be learned, it has never been shown that sulfur in this form is utilized by these bacteria.

In the experiments of Mueller and collaborators (1935 b, 1938) the utilization of cystine was placed on a quantitative basis as a factor in producing normal, heavy growth. Curves were obtained clearly indicating minimal and maximal concentrations. In general, the order of magnitudes found were in good agreement with the quantities used by Braun. The latter appears to have been hampered to a certain extent by the relative

insolubility of cystine, since he found it necessary to add it as a fine suspension. Others have experienced the same difficulty, which in many instances can be avoided by solution of the cystine in dilute HCl. After adding the required amount to the well-diluted medium the pH is suitably adjusted with NaOH, and the presence of the other ingredients of the medium, particularly other amino acids, tends to prevent the cystine from crystallizing.

As to other possible sources of sulfur, cysteine, as would be expected, is effective (Braun, 1938; Locke and Main, 1931; Hosoya, Ozawa and Tanaka, 1933; and others). Locke and Main (1931) find a depression of toxin-formation to occur with cysteine, which they attribute to an alteration of the Cu/Fe ratio in the medium by this amino acid. Scheff and Scheff (1934 a, b) have also observed an irregular diminution of toxigenic action by cysteine, although cystine increased toxin production in their hands. They do not appear to find Locke and Main's explanation involving an effect on Cu to be adequate, but do not offer a better one. Further reference to this matter will be made in the section on toxin.

Braun (1938) has shown that many forms of unoxidized sulfur are capable of replacing cystine in his simple media. These include thio-urea (growth not as good as with cystine),  $\text{Na}_2\text{S}$  and even flowers of sulfur. Braun believes that diphtheria bacilli use sulfur in the form of  $\text{H}_2\text{S}$ , and that only compounds capable of being transformed into this material are suitable for its growth.

These observations have been in part confirmed by Compton and Emerson with the writer (unpublished results) using quantitative methods and the criterion of normal growth. Thioglycolic acid and thiolactic acid gave fairly heavy growth,  $\text{Na}_2\text{S}$  somewhat less and flowers of sulfur relatively poor growth. None were as effective as cystine or cysteine. One could perhaps have anticipated that such would be the case.

Further confirmation of the utilization of  $\text{H}_2\text{S}$  by the diphtheria group is found in the report of Beck (1933), who found an increased rate of growth in an atmosphere of  $\text{H}_2\text{S}$ , and of Lentze (1930) who made a similar observation. The latter believes, however, that it acts as a stimulant and not as a food-stuff.

The only consistent study of the utilization of methionine by

organisms of the diphtheria group appears to be that of the writer. Hosoya, Ozawa and Tanaka (1933) found that it had no effect in their hands. Scheff and Scheff (1934 a, b) observed what they believed to be some increase in the growth but not in toxin when it was added to a medium already containing peptone. In our own work (1935 b, 1935 c, 1938) it was shown that for each strain fully studied, methionine must be added in order to obtain optimal growth. The effect of its omission, while not leading to complete absence of growth, is considerable. Unpublished and incomplete examinations of several other strains gave indications of the same effect. It was found definitely not to replace cystine, nor of course, was cystine alone adequate. A peculiar effect of this amino acid was observed in the case of the first strain to be studied (1935 b), and has not been again encountered. A low concentration of methionine caused a sharp rise in the growth curve, which stopped abruptly as the concentration was further increased, and growth fell to the original level. When histidine, valine and phenylalanine were added to the basic medium, this drop did not occur, and growth increased regularly with the concentration of methionine to the optimum.

#### INORGANIC REQUIREMENTS

One might perhaps anticipate that diphtheria bacilli would manifest a need for the same basic inorganic materials as do other forms of life. Thus, Na, K, Mg, Ca, Fe, and possibly other metals in traces would be expected to be required, and in addition, the inorganic ions Cl and  $\text{PO}_4$ , and perhaps others in minute amounts. The difficulty in connection with obtaining convincing evidence bearing on the matter arises from a consideration of the minute quantities involved, and the practical impossibility of assurance of the absolute purity of materials used. Practically all of the synthetic media which have been tried have contained the usual inorganic salts listed above except Fe; and it is interesting that Uschinsky, in attempting a corroboration of his own earlier observations states (1897) that the addition of a trace of this element improved the growth of the diphtheria bacillus.

Braun and Mündel (1929) seem to have been the first to inves-

tigate the inorganic requirements more closely, and they state that individually neither potassium, magnesium, chloride nor sulfate is required. Mg, Cl and  $\text{SO}_4$  could be omitted simultaneously and "growth" could still result. Five passages were obtained in 29 days on a medium containing only sodium aspartate, cystine, potassium phosphate and sodium acetate. Whether the protoplasm of the diphtheria bacillus is such that it easily readjusts itself to the absence of such universally distributed ions as K, Mg and Cl, or whether the (presumably) scanty growth was able to eke out some kind of an existence on traces of these elements present as impurities, the reviewer does not feel qualified to suggest. The experiments were carried out in quartz-ware using paraffined stoppers, and presumably with care as to purity of materials. Amino acids are, however, notoriously difficult to purify, and may well have supplied traces of elements so widely distributed as the ones in question. Admittedly the purpose of these experiments has been to reduce protoplasm to its lowest terms, and in this, the workers appear to have been completely successful.

Of other experimental work bearing on the matter one finds very little. Wadsworth and Wheeler (1928) reported that a medium containing peptone, glucose, Na, Ca, Mg, Cl,  $\text{SO}_4$  and  $\text{PO}_4$  produced potent toxin only when the Ca and  $\text{PO}_4$  ions were heated together in the presence of peptone. Their explanation, involving a supposed colloidal peptone-calcium phosphate compound appears to be no longer tenable, but the observation is correct, and supplies the most convenient procedure for removing traces of Fe from culture media. The significance of this fact will appear shortly. Later, Wheeler and Mendez (1937) showed that Na, K, Mg and  $\text{PO}_4$  ions were necessary for adequate growth, and stated that Ca, while not essential for growth, must be present for toxin-production.

Our own experiments (1938) in attempting to develop a medium capable of giving "normal" growth have made it possible to show a quantitative need for Mg, K, and  $\text{PO}_4$ , (of the ions so far being considered). It has not been deemed worthwhile to attempt to produce a control-medium free from Na or Cl,

but there have been definite indications in connection with our toxin work that one or both of these would be found essential. The situation as regards Ca appeared to be somewhat different, in that when salts of this metal were omitted, growth took place after a lag of a day or two and became quantitatively as good as when Ca was added, but after a slightly longer time. Without any doubt whatever a certain amount of Ca was present from impurities in other materials. The probabilities are, that with its *complete* elimination, growth would not occur.

We come now to the trace elements, Fe, Cu and Mn. Curiously, these have received the attention of more workers over a period of years than the commoner ones, although most of it has been in connection with studies on toxin-production. We have already noted that Uschinsky observed increased growth with a trace of Fe. Walbum (1921) reported increased yields of toxin after the addition of salts of manganese. Locke and Main (1930, 1931) consider the ratio of copper to iron to be important in toxin-production, and have a theoretical explanation for the phenomenon, which does not appear to be substantiated by the similar experiments of Scheff and Scheff (1934 a, b), nor by certain of our own unpublished experiments. Pope (1932) showed that minute traces of iron increased growth of the diphtheria bacillus and favored toxin-production, whereas the latter process was checked by larger additions. Copper was also found to promote toxin-formation. Strøm (1935) has confirmed both these observations.

The extreme sensitivity of the toxin-producing mechanism of the diphtheria bacillus to traces of Fe, however, seems not to have been appreciated before the work of Pappenheimer (1936) and of Pappenheimer and Johnson (1936). Through an accidental observation that from the same batches of medium, higher titers of toxin were being obtained in certain soft glass Fernbach flasks than in Pyrex glass, the effect was traced to iron, which evidently dissolved out of the soft glass in small quantities. It was found that the medium, prepared according to Wadsworth and Wheeler's (1934) method, was depleted of its Fe by the precipitate of calcium phosphate obtained when heated under

the conditions described above. Without this treatment, the concentration of Fe (present as an impurity in the peptone and other ingredients) is considerably above the optimum, and little toxin is obtained. Its removal by the precipitate is not entirely complete, but usually leaves the concentration somewhat below the optimum. Since the curve of toxin production at varying Fe concentrations is particularly steep on the ascending slope, the traces of Fe dissolving from the soft glass were sufficient to improve the yield noticeably. In general, growth improved with the addition of Fe beyond the optimum concentration for toxin-production, being relatively poor in a medium depleted as thoroughly as possible of its Fe. The concentration of Fe leading to good growth and the best yield of toxin was given by Pappenheimer as 0.000,014 grams per 100 ml.

The writer (1938), investigating the possibility that other metals than Fe might influence growth, fully confirmed the findings of Pappenheimer and Johnson in regard to the growth-promoting effects of Fe in the range described by them. Fairly conclusive experiments were also obtained indicating that Cu, Mn and Zn also somewhat improved growth. These results were in a measure unsatisfactory, because of the difficulty of being certain that the basic medium was sufficiently free from these substances, and must be considered as suggestive rather than conclusive.

In regard to a possible effect of still other substances, it has been reported by Evans, Happold and Handley (1939) that while strains of the diphtheria bacillus belonging to the types *gravis* and *mitis* could be grown with considerable success on a medium whose composition was very similar to that described by the writer, strains classed as *intermediate* grew very sparsely, if at all. Growth of these strains was notably improved by the addition of a solution containing traces of a considerable number of elements, including besides those already mentioned, Al, I, B, Sr, Li, Si, Ti, V and Rb. The effect was not traced to the individual substances involved. The procedure is quite comparable to the use of similar mixtures (so-called A-Z solutions) by plant physiologists.

It therefore appears that for normal heavy growth, the diph-



theria bacillus probably differs in no general way from most other types of living cell.

#### GROWTH ACCESSORIES

Under this heading the writer wishes to include organic substances which take part in promoting normal growth, and which do not fall under the heading of nitrogen, sulfur, or carbon sources. It is, therefore, likely to be a heterogeneous group both in chemical composition and function. The boundaries are not particularly well defined, since tryptophane, for example, could well be included here, rather than under the heading of nitrogen sources. Presumably this amino acid acts, not by supplying nitrogen, but by furnishing a specific grouping of carbon and hydrogen which many strains of the diphtheria bacillus are unable themselves to produce.

Very little definite information on the part played by such a group of substances in the nutrition of these organisms was available until quite recently. The probability that such materials existed and were important constituents of the usual empirical media was expressed by the reviewer in his earliest report dealing with bacterial nutrition (1922). The conviction was repeated and elaborated in discussing the first experiments with *C. diphtheriae* (1933), and some direct evidence was presented concerning them. In 1922 it seemed unfortunate to introduce the term "vitamin" into the situation, since both the conception of accessory factors in the growth of bacteria and the significance of the word "vitamin" were still rather nebulous. Now that both matters are becoming better understood, it is evident that the relationship is very close indeed, and perhaps "accessory factors" and "bacterial vitamins" may be considered to be synonymous.

An occasional suggestion of the involvement of vitamins in the growth of the diphtheria bacillus is to be found in the earlier literature. Leichtentritt and Zielaskowski (1922) reported a favorable effect with lemon juice. Hosoya and Kuroya (1923) found that a vitamin B concentrate improved growth on an otherwise relatively simple medium. Weichart (1928) found that

extracts of typhoid bacilli stimulated growth on a modified Ushinsky medium. Dominici (1928) obtained favorable effects with concentrates of vitamins B and D. Clauberg's diagnostic medium (1931) containing heated blood seems to have been based to some extent on the presence of the X and V factors for *Hemophilus influenzae*. Annok and Buchgraber (1933) and Mustafa (1937 a, b) employed yeast extract, and the latter reports a favorable effect on growth and toxin-formation when crystalline vitamin B<sub>1</sub> was added to a medium containing fresh beef heart infusion and peptic digest of hog stomach. Since none of the strains so far examined appear to be influenced by B<sub>1</sub>, and since his basal medium surely contained the substance, it is difficult to understand the significance of this observation.

Using a basic medium composed of an acid hydrolysate of casein, inorganic salts, and lactic acid, the writer and Subbarow (1937) succeeded in showing that certain components of liver extract (or meat extract) were essential for good growth of a strain of *C. diphtheriae*. That there were at least two substances involved which could be separated by ether extraction of the acidified solution was also shown. One of these was isolated and identified as pimelic acid (Mueller 1937 a). The other fraction was subsequently shown to contain two active materials which were in turn identified as nicotinic acid (Mueller, 1937 b) and  $\beta$ -alanine (Mueller and Cohen, 1937). By the use of suitable amounts of these three substances and an assortment of amino acids, together with salts and lactic acid, extremely heavy growth of this organism was obtained. With Pappenheimer and Cohen (1937) these findings were extended to a strain of Park No. 8, and probably for the first time, toxin of relatively high grade (36 L<sub>t</sub>) was produced on a medium of known composition, free from peptones or polypeptides.

Although these results were shortly confirmed by Evans, Happold and Handley (1939) for a number of strains of types *mitis* and *gravis*, it soon became apparent from their work that other strains could not be grown satisfactorily without still further additions to the medium. Reference to their observations regarding "trace" inorganic elements has already been

made. Later they showed (Evans, Handley and Happold, 1939) that certain type *gravis* strains required pantothenic acid for growth. Mueller and Klotz (1938) had found that pantothenic acid could replace  $\beta$ -alanine for their test strain, the results on the whole being somewhat more satisfactory than in the case of  $\beta$ -alanine alone. It was suggested that this substance was first built up to pantothenic acid, and from the results quoted above it appears that not all strains are capable of carrying out this synthesis. Here is to be found direct confirmation of the theory of "loss of function" outlined in the introductory pages of this review, and further support appears in the same contribution by Evans, Happold and Handley (1939), who found that sterile filtrates of diphtheria bacilli contained substances having the properties of aneurin, riboflavin and coenzymes I and II.

Some further confirmation of the situation as outlined above has been presented by P. Bordet (1939), who identified as nicotinic acid one of the factors in yeast extract which favors growth and toxin-formation.

More recently, facts have been coming to light which indicate that the picture is even more complicated. Because of the luxuriance of growth obtainable on media which are constituted in the above-described manner, Mueller (1939) investigated the possibility of obtaining a solid medium (by the addition of agar) which could be used for diagnostic purposes. It seemed that the absence of vitamin B<sub>1</sub> and of riboflavin, as well as of certain other growth accessories required by the pneumococci and streptococci would make for selectivity and render it particularly suitable. Preliminary experiments showed that in cultures from known cases heavy growth of practically pure *C. diphtheriae* appeared in some instances, whereas not infrequently growth did not occur. A considerable proportion of normal throat cultures also remained without growth, or at most gave only a few scattered colonies. Further investigation revealed that the *size of the inoculum* seemed to be the controlling factor. When plates were streaked, even with strains which grew well on the fluid medium by the usual technic (loopful of pellicle as inoculum) growth was slow in starting, and on the more lightly inoculated

areas of the plate individual colonies did not develop, at least until after two or three days. The indication was clear that a heavy inoculum introduced enough of some still unidentified factor to initiate growth, after which the growing organisms were able to elaborate the material and permit its diffusion into the surrounding medium. It was reported that the addition of whole blood to the medium appeared to supply this deficiency. The substance was in the serum fraction, rather than in the red cells, and remained in solution when the bulk of the serum proteins were removed by heat coagulation.

Snyder, and later Cohen, in the writer's laboratory, have thrown further light on the matter. The former (1940) showed that the effective material was retained by all but the most permeable collodion membranes, and, further, that it could be separated into two components by means of lipoid solvents. They found that not all animal species supplied serum of equal efficacy. Horse and beef serum were particularly suitable, hog and human serum almost without effect. They found that cow's milk offered an abundant and readily available source of material. Cohen and Mueller (1940), and Cohen, Snyder and Mueller (1940) showed that the active substances were precipitated with the casein upon acidification of milk, and could be separated by extraction of this casein, as well as of dry commercial casein, by hot alcohol or cold acetone. The acetone-soluble fraction was extremely abundant in cream and in butter, and has been identified by them as *oleic acid*. This identification appears to be as complete as is possible without the successful substitution of synthetic material. Unfortunately, oleic acid seems not to have been prepared synthetically and our advisors in organic chemistry feel that its attempted synthesis would at this time be attended by great difficulties and considerable uncertainty.

#### NUTRITION IN RELATION TO TOXIN-FORMATION

Insufficient data have accumulated since the recognition by Pappenheimer and Johnson (1936) of the critical nature of the iron effect in toxin-formation to warrant dogmatic generalizations. At the moment it is permissible only to state that this

interference on the part of Fe ions with toxigenicity extends to all strains of *C. diphtheriae* which have been studied from this point of view. These comprise several different "strains" of Park No. 8 (Pappenheimer and Johnson 1936) and a number of heterogeneous strains investigated by Happold (1940). The optimum concentration of iron appears to be about the same, at least of the same order of magnitude, in all cases. An extension of the investigation to a considerable number of other strains would be highly desirable. Naturally it is possible that some will be encountered in which the situation is entirely different.

However, in the light of our present knowledge, it seems to the reviewer that diphtheria toxin may possibly be regarded as an abnormal product of the organism, developed as an emergency mechanism when it is forced to grow under conditions of insufficient iron concentration. Perhaps it may be incorrect to consider it as abnormal, for it is still formed in small amounts (as shown by injection of guinea pigs) even in the presence of quantities of iron considerably exceeding the optimal range (Favorite, unpublished observations). A better statement of the hypothesis, then, would be that it is a substance, in some manner connected with a metabolic function which under normal conditions is carried out chiefly by means of iron-containing enzymes. In the absence of sufficient Fe, the alternative system, involving the toxin molecule, is forced to carry the whole load, and the material is formed in greatly increased amount. If one postulates still further the existence of a second emergency mechanism which does not utilize the toxin molecule, it becomes fairly easy to explain differences in "toxigenicity" between strains, since the response in one or the other auxiliary might well vary with the individual culture.

Perhaps such speculation is not suitable to a review. However, the writer hopes that it may serve to furnish in some measure a reasonable basis from which the older work on toxin-formation in connection with growth may be appraised. From what has already been said, at least one of the traditions with which the subject of toxin-formation is replete is now pretty

well shattered. "Peptone" is no longer to be considered an essential for toxin-formation. A second tradition, namely that growth and toxigenicity are not necessarily related, is both supported and explained, since growth improves (Pappenheimer and Johnson 1936) with the addition of iron beyond the optimum for toxin-production.

It is possible that nowhere can a better illustration be found of the complexity and difficulty of biological experimentation than is afforded by this phenomenon. Innumerable investigators have attempted to study the effect of various substances and agencies on toxin-production. Since almost any manipulation or alteration in composition is practically certain to modify the concentration of iron, it is obvious that many of the effects obtained were due to this fact, not to the one supposed to be under experimentation, and that erroneous conclusions have inevitably been drawn.

At this particular stage in the development of our information it appears unwarranted to review in any detailed way the many papers dealing with toxin-formation as a phase of nutrition of the diphtheria bacillus. Many useful facts have been pretty well established, through numerous repetitions in various laboratories, to a point where it would be absurd to suggest that they should not be considered as beyond criticism. An example of this would be the increased yield of toxin through addition of suitable amounts of maltose. Yet it is perfectly possible to purchase maltose in the market which contains either so much iron or so much readily fermentable material (glucose?) that its use would completely ruin an otherwise satisfactory medium.

The review by Strøm (1935) of factors concerned in toxin production has covered the situation very completely up to the time immediately preceding Pappenheimer and Johnson's work. It is therefore superfluous to undertake a further summary at this time. Since 1935 the whole conception of the matter has been altered by the recognition of the extreme sensitivity of the organism to iron, and by the establishment of the fact that powerful toxin can be formed from amino acids and a few other materials of small molecular size and known constitution. Obvi-

ously no further contributions to the subject merit consideration unless these facts are taken adequately into consideration. It will not be sufficient to assume that Fe concentration remains constant when another point is under investigation. The amounts involved are too small to be estimated accurately by chemical methods now available. The opportunities, moreover, for the accidental introduction of significant amounts of iron into solutions used are extremely great.

At present, the only practicable method of accomplishing control of the iron supply appears to be the rather cumbersome one of purposefully varying the iron through its optimal range for each experimental modification of a basic medium (Mueller and Miller, 1940). This can be done by removing the iron so thoroughly from all materials used that the completed medium is definitely below the optimum for growth as well as toxin-formation. A series of 5 or 6 flasks of similar quantities of such a medium is then prepared, one left without added iron, and small additions of ferrous sulfate or some other iron compound made to the others. If 30 ml. of medium are used in 125 ml. flasks, the amounts of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  which have been found suitable are 3, 6, 9, 12 and 15 gamma. This is conveniently done by using a freshly prepared 0.01 per cent solution, and adding 0.03 ml., 0.06 ml., etc. before using the medium.

Confirmation of these facts has come through the work of Happold (1940), who has reported that several freshly isolated strains of the type *gravis* and one classified as *intermediate* produced moderate amounts of toxin (10 to 15  $\text{L}_t$ ) under these conditions, and gave best results in the range of Fe concentration mentioned above. Further confirmation has come from the experiments of Taylor in the Connaught Laboratories (personal communication).

By the method outlined above, Mueller and Miller (unpublished results) have investigated the effect on toxin-production of most of the individual factors known to be involved in the growth of their strain of Park No. 8. As might be anticipated it is relatively easy to establish an optimal concentration for each individual component of the medium in the presence of

suitable amounts of all others. To arrive at an absolute simultaneous optimum for all constituents in any convincing experimental way involves the manipulation of far too many variables, and could only be accomplished accidentally. It has been possible, however, to formulate a reproducible medium containing acid hydrolysate of casein (Mueller and Johnson, 1940) as the basic source of nitrogen and suitable amounts of all other materials shown to foster the growth of the toxigenic strain, which has consistently yielded  $100 \pm 10$  L<sub>t</sub> of toxin in their hands (Mueller and Miller, 1940). These results have been obtained in both small- and large-scale lots, and the method is entirely suited to practical production.

By way of a summary, it may be of interest to bring together the various facts that have been discussed in the form of specifications for suitable synthetic culture media for the diphtheria bacillus as they have been developed up to the time of writing.

I. A medium giving "detectable growth" with "non-exacting" strains of *C. diphtheriae* (Braun and Hofmeier, 1927).

	gram
Na <sub>2</sub> SO <sub>4</sub> .....	0.5
MgSO <sub>4</sub> or MgCl <sub>2</sub> .....	0.005
KH <sub>2</sub> PO <sub>4</sub> .....	0.05
K <sub>2</sub> HPO <sub>4</sub> .....	0.15
Sodium aspartate.....	0.5
Cystine.....	0.0125
NaC <sub>2</sub> H <sub>3</sub> O <sub>2</sub> .....	0.5
H <sub>2</sub> O to 100 ml.	

II. A medium giving "normal growth" with an "exacting" strain of *C. diphtheriae* (Mueller, 1938).

	gram
L-Cystine.....	0.07
dl-Valine.....	0.2
dl-Methionine.....	0.06
l-Tyrosine.....	0.05
l-Proline.....	0.075
l-Aspartic acid.....	0.5
d-Glutamic acid hydrochloride.....	0.75
KCl.....	0.04
Na <sub>2</sub> HPO <sub>4</sub> .....	0.3



	gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.1
Pimelic acid.....	0.000,015
Nicotinic acid.....	0.000,23
β-Alanine.....	0.000,23
Ethyl alcohol.....	0.7 ml.
d-Lactic acid.....	1.75 ml.
CaCO <sub>3</sub> (in HCl).....	0.02
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.000,5
MnCl <sub>2</sub> ·4H <sub>2</sub> O.....	0.000,25
CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	0.000,5
ZnO (in HCl).....	0.000,25
H <sub>2</sub> O to 100 ml.	

III. A medium yielding, with a Park No. 8 strain, toxin having 36 I<sub>u</sub> per ml., L+ = 0.05 ml. and M.L.D. = 0.000,75 ml. (Pappenheimer, Mueller and Cohen, 1937).

	gram
Glycine.....	0.05
dl-Valine.....	0.1
dl-Leucine.....	0.05
d-Glutamic acid hydrochloride.....	0.5
l-Cystine.....	0.02
dl-Methionine.....	0.02
l-Tryptophane.....	0.01
l-Tyrosine.....	0.01
Pimelic acid.....	0.000,1
β-Alanine.....	0.000,1
Nicotinic acid.....	0.000,2
K <sub>2</sub> HPO <sub>4</sub> .....	0.2
NaCl.....	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.03
CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	0.000,5
Sodium lactate.....	0.74
Maltose.....	0.3
Glucose.....	0.15
CaCl <sub>2</sub> .....	0.006
H <sub>2</sub> O to 100 ml.	

It is clear that developments in the conception of bacterial nutrition are proceeding at a rapid pace, as are those in the field of animal nutrition. The goal of each is the same,—namely a complete chemical understanding of all factors connected with normal growth. Each field has already contributed significantly to the other and this will doubtless continue to be the case. Within the field of bacterial nutrition an even greater relationship

is found as studies on one or another species of organism are continued and compared. The diphtheria group is evidently a particularly fortunate one with which to work. Within itself there appear to be many shades of adaptation or loss of function, in terms of Knight's theory. While this is recognized in Braun's conception of exacting and non-exacting strains, it goes much farther than such a simple and categorical separation into two groups: It is probable that the state of affairs more closely approaches a spectrum, involving a very considerable number of individual functions. These conceivably cover every phase of cell physiology from oxidative and energy-producing to the most complex synthetic functions concerned in the production of all the organic groups and masses of protoplasm itself.

It seems to the writer that up to this time only a beginning has been made toward exploring the tremendous possibilities for advances in knowledge which this group of bacteria supplies. The individual metabolites have been quite completely worked out for a very few strains. These have manifested striking and doubtless significant differences, as well as many similarities. The necessarily attractive goal of practical application has perhaps unduly influenced the direction of certain of the researches. To replace this by the quest for facts of purely scientific import can be done with complete certainty that as such facts accumulate, unexpected interrelationships and significant applications will become apparent at every stage.

Certainly a number of other strains, representing the various types, *gravis*, *mitis* and *intermediate*, should be completely studied. Strains representing Braun's two classifications should be examined to learn whether his differentiation may be fundamental, and the nature of the facts on which it is based. The matter of variation in toxigenicity is still entirely mysterious and is of the utmost importance, theoretical as well as practical. The mechanism of the effect of iron on this phenomenon offers at least one method of approach, and is itself of great interest. If strains are encountered in which the effect of iron is qualitatively or quantitatively different, extremely careful comparisons of these with other strains should certainly be made.

These are a few of the general lines along which it seems that developments may be anticipated. Others will naturally have suggested themselves to the reader. It is not too much to predict that in the next few years very great advances will have been made in our present conception of the whole matter.

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# ENZYMES CONCERNED IN THE PRIMARY UTILIZATION OF AMINO ACIDS BY BACTERIA

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The history of our acquisition of knowledge concerning the utilization of amino acids by bacteria is, in effect, an account of the advancement of methods of bacterial chemistry. The earliest observations of significance were obtained by a chemical study of the products of putrefaction of undefined media by mixed cultures of organisms (3, 4, 5, 84, 14). Such work yielded information concerning the types of compound that could be produced biologically under completely undefined conditions. A step forward was made by the use of pure cultures of organisms (74) and conditions became less obscure with the introduction of synthetic media. A large number of papers deals with the various products of amino acid breakdown that have been obtained by the inoculation of a determined medium, containing a known amino acid, with a pure strain of a known organism, and these have been

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collected and tabulated by Stephenson (86). In much of this work the period of incubation has been long, even as long as three or four months in some cases, and the products isolated chemically at the end of this period, when it is inevitable that conditions should have altered very considerably. Also, it is by no means certain that the organisms have maintained the same enzyme make-up throughout the experiment. Thus we may have recorded as products of amino acid breakdown by certain organisms, substances that are really produced in stages at different times during the incubation period, under different environmental conditions and by what are, biochemically speaking, different organisms.

The next advance was made when it was realized that it is necessary to study the metabolism of an organism, not as an integral whole, but as a collection of reactions catalyzed by specific enzymes contained in the bacterial cell. Such a study is impossible while organisms are growing in culture, as any change studied in the growth medium may be due to catabolic or anabolic reactions, or to both. To avoid this ambiguity in interpretation, the technique of the washed suspension of bacteria was evolved. By this method, experiments several hours in duration can be carried out in the absence of cell growth. This technique was first used for suspensions of *Escherichia coli* by Quastel and Whetham in 1925 (76, 77). By the use of washed suspensions together with the methylene blue, manometric and other techniques of biochemistry, not only can an idea of the relative rates and of the quantitative nature of bacterial attack on amino acids be obtained, but also the conditions of pH and temperature under which the enzymes involved are active can be investigated, as can also the growth conditions necessary for the optimal formation of such enzymes. Examples of such advances made in the field of amino acid metabolism are detailed below.

The washed suspension technique allows of investigation of the properties of enzymes as they exist in the bacterial cell but such investigation is necessarily restricted by the presence and action of other enzymes in the cell, by the permeability of the cell membrane towards the enzyme substrate, etc. A detailed ac-

count of the properties of any particular enzyme can only be given after it has been obtained in a cell-free condition. Until recently bacterial chemistry has lagged behind other branches of biochemistry in this respect owing to the difficulty of breaking up the bacterial cell with the general liberation of its enzymes; but since the production of the wet-crushing bacterial mill of Booth and Green (18) an increasing number of cell-free bacterial enzymes are being studied (38, 44). Several enzymes involved in the metabolism of amino acids have been obtained in a cell-free state by this and other methods and will be discussed below. The ideal of a crystallized bacterial enzyme of this nature has not, up to the time of writing, been realized.

Bacteria are known to utilize amino acids as (1) a source of nitrogen (and possibly carbon) for cell-growth and multiplication and (2) as a source of energy. Such utilization involves both catabolic and anabolic changes, and most of our more exact knowledge up to the present is related to the former, so that it will be chiefly with the catabolic type of change that this paper will deal.

#### THE BREAKDOWN OF AMINO ACIDS BY BACTERIAL ENZYMES

If we consider the immediate degradation of the amino acid molecule by bacterial enzymes, four ways of attack appear available:

1. Removal of the amino group in the  $\alpha$ -position, or deamination.
2. Removal of the carboxyl group, or decarboxylation.
3. Deamination accompanied by decarboxylation.
4. Splitting of the molecule into smaller molecules other than by deamination or decarboxylation.

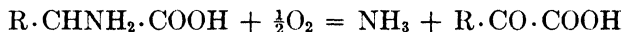
A possible product of reaction 4 is an amino acid of smaller molecular weight but, in general, the products of these methods of attack are no longer amino acids and hence need not be followed further in a study of enzymes whose substrates are specifically amino acids. Thus it is intended in this review to restrict the discussion to those enzymes immediately concerned

with the initial attack on amino acids and to omit further consideration of the fate of the products unless such consideration is essential for the understanding of the primary reaction.

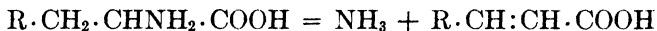
1. REMOVAL OF THE AMINO GROUP FROM THE  $\alpha$ -POSITION:  
DEAMINATION

The enzymes involved in this type of breakdown are usually termed "deaminases" but it must be realized that the reaction catalyzed by a "deaminase" may actually consist of more than one step, each catalyzed by a separate enzyme, as in the case of the "glutamic acid deaminase" discussed below. Deamination may be brought about in a variety of ways, each resulting in a different product:

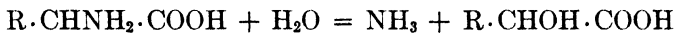
a. Oxidative deamination to give a keto acid



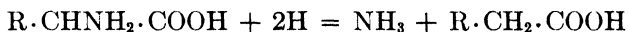
b. Desaturation deamination to give an unsaturated fatty acid



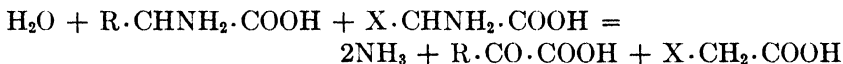
c. Hydrolytic deamination to give a hydroxy acid



d. Reductive deamination to give a saturated fatty acid



e. Mutual oxidation-reduction between pairs of amino acids



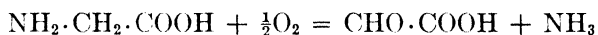
The occurrence of each of these types of reaction, together with others (*f*) which have not been fully classified, in the metabolism of bacteria will be dealt with separately. These will be followed by (*g*) a consideration of the factors influencing bacterial deamination.

(a) *Oxidative deamination*

The work of Krebs (68, 69, 70, 71, 72) has established that mammalian kidney and liver oxidatively deaminate the majority

of amino acids that are metabolized by these tissues. The demonstration of oxidative deamination involves showing (a) the liberation of  $\text{NH}_3$  in the presence of  $\text{O}_2$  or some oxidizing agent only, (b) the isolation of the keto acid formed and, if possible (c) that the consumption of  $\text{O}_2$  is equivalent to the  $\text{NH}_3$  evolved when the metabolism is stopped at the keto acid stage.

Stephenson and Gale (87) showed that washed suspensions of *E. coli* will deaminate glycine, *dl*-alanine and *l*(+)-glutamic acid aerobically but not anaerobically. As the work was designed for another purpose, no attempt was made to isolate the deamination product, but Janke and Tayenthal (60) studied the reaction with glycine in detail. They showed that the deamination is carried out by *E. coli*, *Proteus vulgaris*, *Pseudomonas fluorescens*, and *Bacillus mycoides* aerobically but not anaerobically unless some oxidizing agent such as *m*-dinitrobenzene is present. The aerobic deamination was performed in the presence of 2,4-dinitrophenylhydrazine which acts as a fixative for keto acids, and a substance was isolated having the color reactions and melting point of the glyoxylic acid derivative. Thus the deamination of glycine by the bacteria studied may be described by the equation:



The pH optimum for the reaction was between 7.5 and 8, and this appears to be a general finding for all enzymes of this class so far studied (43, 87). (See fig. 1.)

Aubel and Egami (12) demonstrated the deamination of *dl*-alanine by an unidentified soil organism and showed that the reaction will occur aerobically but not anaerobically except in the presence of nitrate. Simon's color test for pyruvic acid proved positive when applied during the reaction and from this fact together with the effects of various inhibitors, these workers assume that the deamination process is an oxidative one, involving dehydrogenation followed by hydrolysis of the imino acid so formed. The proof is not rigid, but some unpublished work carried out in this department with washed suspensions of *E. coli* shows that this organism carries out oxidative deamination of *dl*-alanine. It has already been shown (87) that a washed sus-

pension prepared from the organism grown on the surface of broth-agar (tryptic digest of casein) will deaminate *dl*-alanine aerobically at pH 7.5. If the suspension is first treated with toluene and the toluene then removed, the deamination rate is halved and a positive Simon's nitroprusside test shows the accumulation of pyruvic acid. A large-scale experiment was set up

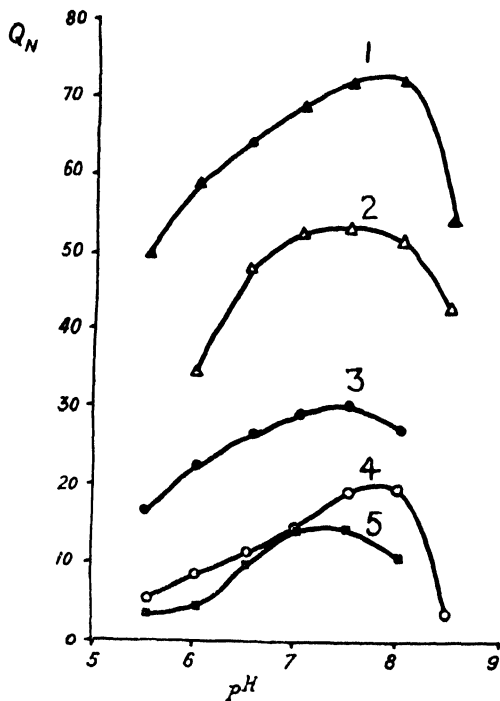
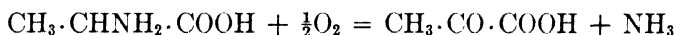


FIG. 1. VARIATION WITH pH OF THE ACTIVITY OF VARIOUS DEAMINASES IN WASHED SUSPENSIONS OF *ESCHERICHIA COLI*

1, *dl*-Serine; 2, *l*-aspartic acid; 3, *dl*-alanine; 4, *l*-glutamic acid; 5, glycine (38, 43, 87)

with toluene-treated organisms, alanine and phosphate buffer at pH 7.5 and incubated aerobically until a manometric control showed that about 60 per cent of the alanine was oxidized (on the assumption that  $\frac{1}{2}$  mol.  $O_2$  would be taken up for each mol.  $NH_3$  liberated). The organism was centrifuged off and the centrifugate analyzed. Pyruvic acid was isolated as its 2,4-dinitro-

phenylhydrazone and estimated by the use of a carboxylase preparation from brewer's yeast. Also the ammonia-N was estimated by the Parnas technique and the amino-N by the van Slyke method. Table 1 gives the full analysis. These results prove that the deamination of *dl*-alanine by *E. coli* is accomplished according to the equation:



Attempts to demonstrate the presence of an alanine-dehydrogenase in washed suspensions of *E. coli* or in crushed cells have not so far been successful so that no evidence has as yet been adduced for the existence in bacteria of an alanine-dehydrogenase-coenzyme as demonstrated for the kidney *d*-amino-acid-oxidase (25, 90, 98, 99).

TABLE 1

*Products of deamination of dl-alanine by toluene-treated suspensions of E. coli*

Total theoretical N.....	342 $\mu$ l	
Amino-N.....	197 $\mu$ l	} 347 $\mu$ l
Ammonia-N in centrifugate.....	150 $\mu$ l	
Pyruvic acid.....	153 $\mu$ l	

In the case of the *l*(+)-glutamic acid deaminase of *E. coli*, Adler and his co-workers (9) have been able to extract the "enzyme" from the cell by a modification of the method used by Stephenson (85) for the extraction of lactic dehydrogenase. The extracted enzyme reduces methylene blue in the presence of glutamic acid, requiring the presence of coenzyme II for its activity. The reduction time is increased some 3 to 5 times by the presence of either ammonium chloride or ketoglutaric acid, while the combined presence of these substances increases the reduction time 7 to 8 times. These results suggest that the enzyme involved is a dehydrogenase and that the reaction is reversible. This is confirmed by spectrometric studies of the reduction of coenzyme II in the presence of the enzyme and *l*-glutamic acid, and of the oxidation of dihydro-coenzyme II in the presence of the enzyme and ketoglutaric acid together with

ammonia,—neither being effective alone. The general scheme that has been worked out as a result of these studies is

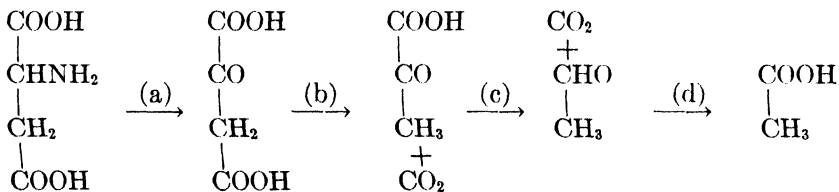
- (a) Glutamic acid + coenzyme  $\rightleftharpoons$  Iminoglutaric acid + dihydro-coenzyme;  
 (b) Iminoglutaric acid +  $\text{H}_2\text{O} \rightleftharpoons$  Ketoglutaric acid +  $\text{NH}_3$   
 (c) Dihydro-coenzyme +  $\frac{1}{2}\text{O}_2 =$  Coenzyme +  $\text{H}_2\text{O}$

Thus the amino acid is deaminated in two steps, each being reversible, and oxygen is taken up for the reoxidation of the dihydro-coenzyme formed in the first step. Adler and his co-workers (7, 8, 9) believe this reversible reaction is of importance in shedding light on the problem of amino acid synthesis in various tissues.

Klein (64) in a recent paper has shown that washed suspensions of *Hemophilus parainfluenzae* oxidize *l*(-)-aspartic and *l*(+)-glutamic acids with the liberation of ammonia and the formation of acetic acid, according to the equations:



By a study of the metabolism of possible intermediate compounds, Klein has established the probable course of the oxidation. For aspartic acid, the reaction proceeds:



The reactions (a) and (d) require the presence of either coenzyme I or II, the exact requirement not being established. Thus it appears from this work that the first step in the breakdown of the dicarboxylic amino acids by *H. parainfluenzae* is an oxidative deamination, probably proceeding in stages similar to those elucidated by Adler (9).

Woods and Clifton (104, 105) have shown that *Clostridium*

*tetanomorphum* breaks down certain amino acids with the evolution of  $H_2$ , and it may be that this is a form of oxidative deamination without the use of either oxygen or a hydrogen acceptor. However, in most cases, the other products have not been determined and in no case as yet is it possible to state by what particular process the ammonia is primarily liberated from the amino acid molecule.

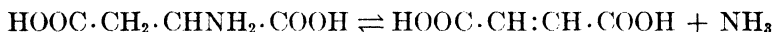
Bernheim, Bernheim and Webster (15) extended their work on deamination by animal tissues to the deamination and oxidation of amino acids by resting *Proteus vulgaris*. They showed an oxygen uptake in the presence of the washed suspension and the natural isomers of leucine, phenylalanine, methionine, serine, alanine, proline, tyrosine and tryptophane and this  $O_2$  uptake was accompanied by the evolution of ammonia. No attempt was made to isolate the immediate products of the deamination process or to check the oxidation process at this point so that it is impossible to tell from their results whether the oxygen taken up is involved in an oxidative deamination or in the oxidation of the deamination products. In some cases more than 1 atom of  $O_2$  was taken up per mol. amino acid oxidized so that some of this gas at least must be involved in the later oxidative changes. Also one or two similar experiments carried out with washed suspensions of *E. coli* showed that this organism deaminates aerobically serine, alanine and glycine, but it has later been shown (43) that the deamination of serine will also take place anaerobically. Thus this work, and similarly that of Webster and Bernheim (100) on *Pseudomonas aeruginosa* does not indicate the exact nature of the deamination process but yields useful information concerning the general utilization of amino acids under aerobic conditions by the organisms studied.

#### (b) Desaturation deamination

Quastel and Whetham (76) showed that *E. coli* brings about the reduction of fumaric acid to succinic acid and also sets up an equilibrium between fumaric and *l*-malic acids. Thus the presence of malic acid retards the reduction of methylene blue by succinic acid in the presence of the washed suspension. As-



partic acid also retards this reduction (77) and this observation led Quastel and Woolf (78) to investigate whether aspartic acid is deaminated by *E. coli* to fumaric acid. The first suggestion that some such reaction takes place was given by Harden in 1901 (51) who showed that *E. coli* grown in glucose broth will deaminate aspartic acid with the final formation of succinic acid. Quastel and Woolf (78) found that the deamination of *l*-aspartic acid is carried out by washed suspensions of *E. coli*, the product in the absence of any inhibitor being succinic acid. If however an inhibitor such as toluene is present, then the rate of the deamination process is not affected in the initial stages; but the process does not go to completion, reaching, instead, an equilibrium mixture of aspartic acid, fumaric acid and ammonia. Further, if the correct proportions of fumaric acid and ammonia are incubated in the presence of the cells and toluene, then aspartic acid is synthesized until the same equilibrium mixture is produced. Thus we have the reversible deamination process:



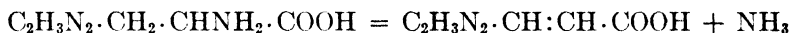
In the absence of inhibitors, the fumaric acid is reduced to succinic acid and the equilibrium conditions are upset. Woolf (107) also showed that fumarase present in the organism produces malic acid from the fumaric acid and that the final equilibrium mixture is not a simple aspartic acid-fumaric acid-ammonia system but one containing malic acid as well.

The existence of the desaturation deaminase was proved by Virtanen and Tarnanen (97) who obtained from *Pseudomonas fluorescens* in a cell-free state (by extracting the dried cells with toluene-water) the aspartase responsible for the production of fumaric acid from aspartic acid. The filtered extract contained fumarase and aspartase, but the former was removed by tryptic digestion, leaving a preparation with which the properties of the cell-free aspartase could be studied.

Gale (38) investigated the deamination of *l*-aspartic acid by washed suspensions of *E. coli* and by a study of the loss and recovery of activity of the suspensions on standing under various conditions, discovered that the rate of deamination is greatly

increased by the addition of small amounts of adenosine or inosine. This effect had not been noticed for the aspartic acid enzymes previously studied. The enzyme was obtained in a cell-free condition by the use of thick washed suspensions crushed in the mill of Booth and Green (18). It soon became obvious that there were at least two enzymes in the cell-free juice so obtained which would deaminate aspartic acid anaerobically. Simple ammonium sulphate fractionation led to the separation of two enzymes: one stable to incubation with toluene and unaffected by the presence of adenosine; the other, inactive in the absence of some coenzyme which could be replaced *in vitro* by adenosine, and completely inactivated by toluene treatment. The first of these enzymes was presumably the aspartase of Quastel and Woolf (78) and was therefore renamed "aspartase I" while the other, hitherto unstudied, enzyme was named "aspartase II." Both enzymes are optimally active at pH 7.5. Since however the simple fractions containing the separate enzymes contained fumarase, and no method of separating aspartase II and fumarase could be found, owing to the greater sensitivity of the former, it is impossible to state the immediate product of the deamination accomplished by aspartase II,— a mixture of fumaric and malic acids being obtained from aspartic acid by the action of the fraction containing the latter enzyme.

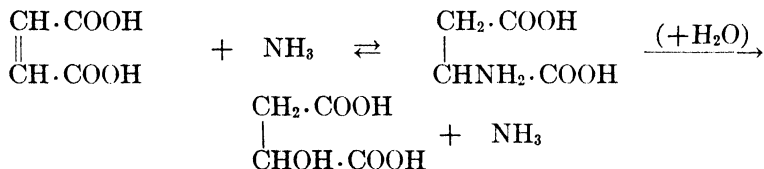
An earlier case of desaturation deamination is recorded by Raistrick (79) who obtained urocanic acid from *l*-histidine:



The urocanic acid was obtained by the action of *E. coli*, *Eberthella typhosa*, *Salmonella paratyphi*, *S. enteritidis* and *Shigella dysenteriae* respectively on histidine in Ringer's solution. The author states that growth in this medium was poor and that this difficulty was overcome by using a heavy inoculum from several agar slopes. Since however the organisms were in culture over a period of 30 to 40 days, it is not possible to state with certainty whether the urocanic acid is a primary product of desaturation deamination or is produced by a secondary action upon the product of deamination by some other process.

(c) *Hydrolytic deamination*

Woolf (107) showed that the deamination of aspartic acid by *E. coli* suspensions in the presence of toluene results in an equilibrium mixture of aspartic, fumaric and malic acids, the malic acid being produced by the action of fumarase on the fumaric acid produced from aspartic acid by aspartase I. Following the fractionation by Gale (38) of the aspartases of *E. coli* into two separate enzymes, Virtanen and Erkama (92) reported the separation of two aspartases from *Pseudomonas fluorescens* using a method of fractionation involving acetic acid. Their preparation did not contain fumarase as it would not carry out the formation of malic acid from fumaric acid in the absence of ammonia. However, in the presence of ammonia, malic acid was formed from fumaric acid and Virtanen explains this as follows: The preparation contains two enzymes acting upon aspartic acid, one deaminating it to form fumaric acid and one to form malic acid, the former being reversible but not the latter. Thus when fumaric acid is incubated with the preparation in the presence of ammonia it is first converted to aspartic acid by reversal of "aspartase I" and the aspartic acid is then irreversibly deaminated to malic acid by the other enzyme present.



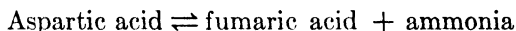
Both enzymes are active in the presence of toluene. It seems probable that the postulated reversible enzyme in this work is aspartase I but whether the other enzyme bears any relation to aspartase II is sheer conjecture. In any case, it is doubtful whether it is legitimate to apply results obtained with one organism to any other. If Virtanen has established the existence of an enzyme forming malic acid directly from aspartic acid, this will be the first positive demonstration of a hydrolytic deaminase.

There are several references to hydroxy acids being isolated from growth experiments involving certain amino acids (11, 54,

82, 83) but there is no evidence in these cases that the acid is a product of hydrolytic deamination.

(d) *Reductive deamination*

Cook and Woolf (24) carried out an investigation designed to determine the distribution of the enzymes responsible for the production of (a) fumaric acid, and (b) succinic acid from aspartic acid. They used 11 organisms representative of the strict aerobes, strict anaerobes and facultative anaerobes. Washed suspensions of all 11 deaminated *l*-aspartic acid to succinic acid when incubated at 37° anaerobically. This reaction was completely inhibited by the presence of toluene. The four facultative anaerobes all effected the reaction:

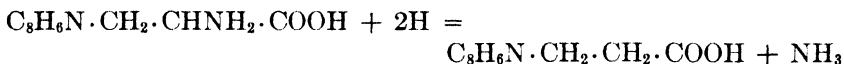


giving the same equilibrium constant as that obtained for the aspartase of *E. coli*. However, none of the strict aerobes or strict anaerobes showed any trace of this reaction; and it must be assumed that these latter organisms carry out a simple reductive deamination:



No evidence could be obtained for the reversibility of this reaction.

Hopkins and Cole (58) showed the production of  $\beta$ -indole-propionic acid from tryptophane by *E. coli* growing in culture. Woods (101) studied the production of indole from tryptophane by thick washed suspensions of *E. coli* and found that the reaction only took place under strongly aerobic conditions. The product of the anaerobic attack proved to be  $\beta$ -indole-propionic acid and was formed quantitatively.



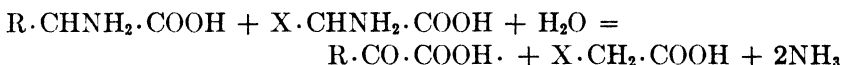
The hydrogen necessary for this reaction comes presumably from the endogenous hydrogen-donators of the cell. No indole is formed from  $\beta$ -indole-propionic acid in the presence of washed suspensions under otherwise optimal conditions for indole pro-

duction, from which it is concluded that the acid does not form an intermediate in the aerobic production of indole from tryptophane.

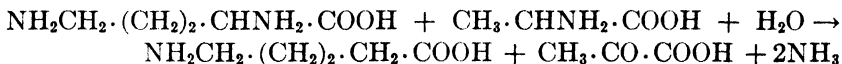
The production of saturated fatty acids from corresponding amino acids by organisms growing in culture has been reported by various authors (19, 20, 65).

(e) *Mutual oxidation-reduction of pairs of amino acids*

Stickland (88, 89) showed that washed suspensions of *Clostridium sporogenes* activate certain amino acids as hydrogen-donators and others as hydrogen-acceptors so that coupled reactions take place between pairs of them, resulting in their deamination. The examination of the function of the amino acids in this respect was continued by Woods (103); and the results of these two workers show that the following amino acids act as H-donators: the natural isomers of alanine, valine, leucine, phenylalanine, cysteine, serine, histidine, aspartic acid, and glutamic acid; and the following as H-acceptors: glycine, proline, hydroxyproline, ornithine, and arginine. The general type of reaction is probably of this nature:



The products of the complete reaction have not been identified in all cases. However, the reaction between alanine and ornithine probably follows this scheme as the products have been identified as  $\delta$ -aminovaleric acid, ammonia, acetic acid and  $\text{CO}_2$ , the latter two arising from the alanine probably by way of the intermediate production of pyruvic acid:



Similarly the reaction between glycine and alanine gives rise to 2 molecules of acetic acid,  $\text{CO}_2$  and  $\text{NH}_3$ . There are presumably several enzymes involved in this type of reaction, one of which is an amino acid dehydrogenase similar to that postulated in the oxidative deamination discussed above but, whereas in that case

the "activated" hydrogen is taken up by the coenzyme, in this case the H-acceptor is another amino acid molecule.

Hoogerheide and Kocholaty (57, 66) have shown that *C. sporogenes* can activate and utilize  $H_2$  and, using this as a method for the investigation of H-acceptors, claim that some substrates can act as either H-donators or -acceptors so that intermolecular reactions occur in which one molecule is oxidized and another reduced. Clifton (23) has shown that *Clostridium botulinum* also utilizes amino acids by means of the Stickland reaction.

(f) *Unclassified deamination processes*

The work of Bernheim and his co-workers (15, 100) on the aerobic breakdown of amino acids by washed suspensions of various organisms has already been discussed. This work is of interest in particular for the light it gives on the optical specificity of the bacterial attack on amino acids. The organisms used were *Proteus vulgaris* and *Pseudomonas aeruginosa* and their action in washed suspension on 13 amino acids was studied. Both organisms attack both isomers of alanine and serine, and the latter deaminates also both isomers of tyrosine and proline; in all other cases only the natural *l*-isomer is attacked. Gale and Stephenson (44) have shown that washed suspensions of *E. coli* will deaminate *dl*-serine anaerobically at an optimum pH of 7.5. The isomers are attacked at different rates but the product of the deamination process has not been identified.

A series of papers by Desnuelle *et al.* (26, 27, 28) has dealt with the deamination of cysteine and cystine by washed suspensions of *E. coli*. Cysteine is attacked anaerobically with the liberation of  $NH_3$  and  $H_2S$  in equimolecular quantities, the enzyme responsible for the liberation of the  $H_2S$  ("cysteinase") being strictly adaptive in the sense of Karström (61). Cystine is attacked in the presence of a reducing agent, or during the cofermentation of glucose, so that it is first reduced to cysteine. The other products of the breakdown have not yet been reported. A further paper (29) deals with the anaerobic degradation of cysteine and cystine by *Propionibacterium pentosaceum* and reveals marked differences between the modes of attack on these amino acids by this organ-

ism and *E. coli*. For instance whereas the cysteinase of *E. coli* is strictly adaptive, partially inhibited by the presence of glucose and specific for the natural isomer of cysteine, the enzyme of *Propionibacterium pentosaceum* is not adaptive, is accelerated by the presence of glucose and shows no optical specificity. Tarr (91) has shown that cystine is disrupted anaerobically at pH 7.8 by washed suspensions of *Proteus vulgaris* with the formation of ammonia, hydrogen sulphide, hydrogen, carbon dioxide and acetic acid. Washed suspensions of *Clostridium tetanomorphum* attack *l*-cystine with the liberation of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ ,  $\text{CO}_2$  and unidentified products (Woods and Clifton, 101, 102).

The breakdown of tryptophane by bacteria has been the subject of many papers. It has been mentioned above that Woods (101) has shown the reductive deamination to  $\beta$ -indolepropionic acid by *E. coli* under anaerobic conditions and this has been confirmed by Majima (73). Under aerobic conditions, tryptophane is converted quantitatively to indole (52, 101) but, up to the time of writing no attempt to identify the intermediate products has met with success. Woods (101, 102) showed that ammonia is liberated quantitatively and 5 atoms of oxygen are taken up during the complete conversion to indole by washed suspensions of *E. coli*. He was unable to show indole formation under similar conditions from  $\beta$ -indolealdehyde,  $\beta$ -indolecarboxylic acid,  $\beta$ -indoleacetic acid,  $\beta$ -indolepropionic acid or  $\beta$ -indoleacrylic acid. On the other hand,  $\beta$ -indolepyruvic acid gave a small (10%) production of indole in the presence of ammonia, which suggests that there is first a resynthesis to tryptophane. Similar conclusions have been drawn by other workers (37, 73, 81). Happold and Hoyle (49) obtained a non-viable "tryptophanase" from *E. coli* cells which forms indole from tryptophane under aerobic conditions but will not react with  $\beta$ -indolealdehyde,  $\beta$ -indolepropionic acid,  $\beta$ -indoleacetic acid, or  $\beta$ -indolepyruvic acid. A later paper by these workers (13) deals with the groups in the side-chain of the tryptophane molecule essential for the action of the tryptophanase. Their results indicate that the breakdown to indole requires (a) a free carboxyl group, (b) an unsubstituted  $\alpha$ -amino group and (c) a  $\beta$ -carbon atom capable of oxidative

attack. They suggest a tentative scheme for the breakdown which involves a reductive fission of the molecule, but this awaits experimental confirmation.

Woods and Clifton (101, 102) have described an anaerobic breakdown of certain amino acids by washed suspensions of *Clostridium tetanomorphum* which results in the liberation of ammonia and hydrogen; this work has been mentioned above as demonstrating a possible form of anaerobic oxidative deamination

(g) *Factors influencing bacterial deamination*

*"Age of culture."* Wooldridge and co-workers (106) first showed that the activity of certain bacterial dehydrogenases varies markedly with the age of the culture from which the cells have been harvested. This applies also to many of the deaminases: Gale and Stephenson (43) showed that serine-deaminase may vary in activity from  $Q_N = 200$  for a 6-hr. culture to  $Q_N = 1100$  for a 12-hr. culture, the period of maximum activity roughly coinciding with the cessation of active cell-division. In the case of aspartase (38) this variation in activity with "age of culture" is due to a change in the properties of the growth medium brought about by the metabolic activities of the cells.

*Anaerobiosis and aerobiosis.* Stephenson and Gale (38, 43, 87) have investigated the effect of growing cultures of *E. coli* under varying conditions of aerobiosis on the deaminase activities of the washed suspensions. Generally speaking, their results show that the oxidative enzymes are formed best under strongly aerobic conditions while the formation of the anaerobic deaminases is favored by anaerobic growth conditions.

*Effect of the presence of glucose during growth.* Kendall and his co-workers, in a series of papers from 1912 to 1922, studied the production of ammonia by several bacterial species when growing in protein digests and showed that this production is greatly checked and, in some cases, completely inhibited by the presence of glucose in the growth medium. They interpreted this as due to a "sparing" action exerted by the carbohydrate on the deamination of proteins, believing that in the presence of a readily available source of carbon and energy, the organism decomposed



less nitrogenous material. Raistrick and Clark (80) pointed out that ammonia is not only a product of the decomposition of proteins but also a source of nitrogen for growth, so that while, in the protein digest medium, the ammonia produced is in excess of that required for cell synthesis, it may be possible that in the presence of additional carbohydrate, this excess is used up in increased cell production. In investigating this point they followed the growth of various bacterial species in synthetic media containing tryptophane or tyrosine with and without glycerol. They estimated the ammonia-N, amino-N, total-N and "synthesized-N" (= cell-N) after periods of 19 to 51 days. They showed that the presence of glycerol in the medium (a) increases the amount of cell-N formed and (b) decreases the amount of ammonia-N formed, and stated that "we believe that carbohydrate, far from having a protein-sparing effect, actually enables the bacteria to utilize more protein or protein products than they would in the absence of carbohydrate." A detailed investigation and discussion of this point has been carried out by Waksman and Lomanitz (97a) who come to much the same conclusions but point out that "a living being derives its energy from a substance which is most available to it and which may be specific for a particular organism."

Happold and Hoyle (49, 50) investigating the production of indole from tryptophane by *E. coli* (see above) have described the preparation of a non-viable "tryptophanase" from cultures of the organism and report that such preparations made from cultures grown in the presence of glucose are inactive, whereas active preparations are not inhibited by the presence of glucose. These workers adduced evidence that tryptophanase is an adaptive rather than a constitutive enzyme, and Fildes (35) reports that, although the organism grown in the absence of tryptophane contains a small amount of tryptophanase, the presence of this amino acid in the growth-medium considerably increases the amount of enzyme in the cells. He further found that it was this "adapted" portion of the tryptophanase which is inhibited by the presence of glucose in the growth medium,—the constitutive part remaining unaffected. Happold and Hoyle (50) con-

sider that the "carbohydrate affords a readier source of energy for *B. coli* than does the tryptophane so that the bacillus does not require to elaborate a mechanism which it has evolved for normal life."

Stephenson and Gale (87) investigated the effect with regard to the deaminases of glycine, alanine and glutamic acid in washed suspensions of *E. coli*. They showed that the presence of glucose has little or no effect upon the actual deamination process by washed suspensions grown in tryptic digest of casein, so that once the enzyme make-up of the cell has been completed, the glucose has no sparing-effect or inhibition-effect on the deamination. If, however, glucose is added to the *growth* medium, then the resulting washed suspension has less than 5 per cent of the deaminase activity of similar suspensions of bacteria grown in the absence of carbohydrate. The effect is not due to anaerobiosis produced by fermentation gases as the effect is not altered by bubbling the growth medium with oxygen. These observations were later extended to the deamination of *dl*-serine (43) and of *l*-aspartic acid (38) and it was suggested that the principal effect of glucose is to inhibit the formation of the deaminases in question during growth. Whether the glucose acts as such or whether its action is due to the acids produced from it during the fermentation, as is suggested by the work of Berman and Rettger on proteolytic enzymes, is not certain. In the above work (87) the pH of the medium was maintained near neutrality during growth by the addition of chalk to the medium, but it is doubtful whether such a device is effective in controlling the pH in the immediate vicinity of a rapidly fermenting organism. In view of the recent work, to be discussed later, on the conditions under which the amino acid decarboxylases are formed in bacteria, it appears probable that the presence of carbohydrate in the medium, by altering the pH in the vicinity of the organism and consequently the ionization of the substrate, may change the focus of the attack on the amino acid from the amino group to the carboxyl group. The question would be clarified by a study of the formation of the various deaminases during growth of organisms in non-carbohydrate media adjusted to various pH values.

*Existence of co-deaminases.* Mention has been made of the finding (9, 64) that the *l*(+)-glutamic acid dehydrogenase of *E. coli* and *Hemophilus parainfluenzae* requires the presence of coenzyme II for its activity. The results obtained with animal tissues (90, 98, 99) would lead us to expect that other bacterial oxidative deaminases will be resolved into dehydrogenases which will only be active in the presence of certain coenzymes, but no further evidence along these lines has yet been reported.

In the case of the anaerobic deaminases, Gale and Stephenson (43) showed by a study of the loss and recovery of activity towards *dl*-serine of washed suspensions of *E. coli* that the *dl*-serine deaminase probably requires a coenzyme. This postulated coenzyme appears to exist in a phosphorylated and non-phosphorylated form and in a reduced and an oxidized form, the phosphorylated and reduced form being active as codeaminase. Somewhat similar investigations of the activity of aspartase II of *E. coli* both in a cell-free condition and in the bacterial cell showed (38) that this deaminase requires a coenzyme which can be replaced *in vitro* by adenosine or inosine.

## 2. REMOVAL OF THE CARBOXYL GROUP: DECARBOXYLATION

Ellinger (32) and Ackermann (3, 4, 5) were the first to demonstrate the formation of various amines during bacterial putrefaction. Their method was to inoculate a synthetic medium containing salts, peptone, glucose and an amino acid, with decomposing pancreas and then, after an incubation of some weeks, to isolate the amine from the medium. In such a manner Ackermann demonstrated the formation of putrescine and  $\delta$ -aminovaleric acid from arginine, and of histamine, cadaverine and  $\gamma$ -aminobutyric acid and  $\beta$ -alanine from their corresponding amino acids. This work is typical of the early investigations (1, 32, 33) showing the bacterial formation of amines without giving information concerning the processes, organisms or enzymes involved.

An advance was made by the use of pure strains of organisms growing in synthetic media. Thus Berthelot and Bertrand (16, 17) isolated a "*Bacillus aminophilus intestinalis*" (said to be

related to *Klebsiella pneumoniae*) which proved capable of forming histamine, tryptamine and tyramine. Strains of *E. coli* and *Proteus vulgaris* are reported to form tyramine from tyrosine (82) and isoamylamine from leucine (11). The formation of putrescine by *E. coli* growing in a medium containing arginine as sole source of nitrogen has been claimed by Akasi (10) and Hirai (56), while the formation of histamine has been the subject of many papers (55, 62, 63, 75). In all this work the general technique has been the inoculation of a synthetic medium containing inorganic salts, the amino acid in question and either glycerol or carbohydrate, with a pure strain of the organism. This is followed by a lengthy incubation and chemical isolation of the amine product. This method is usually not quantitative, and the long incubation may entail considerable variations in the enzyme make-up of the organism as the constitution of the medium alters.

Koessler and Hanke (65) studied the formation of histamine by a "colon bacillus" isolated from a case of cystitis and used an extraction method to remove histamine from the culture medium followed by colorimetric estimation. They showed that whenever the amine is produced, the medium first becomes distinctly acid and that "histamine is never produced except in the presence of an easily available source of carbon such as glycerol or glucose." This conclusion was later (46, 47, 48) found to apply to many strains of "colon bacilli" (isolated from feces) which were able to form histamine from histidine. Some strains of these organisms also produced tyramine when grown in a medium containing tyrosine, but it was found that the organisms which formed tyramine would not form histamine and *vice versa*. After an improvement of the method (31) of extraction of histamine from culture media, Eggerth (30) carried out a detailed investigation of the production of histamine by many strains of *E. coli*, *Salmonella spp.*, *Eberthella spp.*, *Aerobacter spp.*, etc. The organisms were grown in various determined media containing inorganic salts, glucose and, in some cases, asparagine or peptone to assist growth. In favorable conditions histamine production began within 24 hours and continued for 4 or 5 days. Experiments in which the pH of the medium was adjusted during growth

showed that histamine is produced most rapidly, in most cases, between pH 5.0 and 5.5 and, for most organisms, not at all at reactions more alkaline than pH 6.5. The work showed also that the temperature of incubation markedly affects the histamine formation, some organisms having an optimal temperature below 30° and others around 37°. The results reveal the large number of intestinal organisms capable of producing histamine; but as they deal with organisms growing in culture, they do not indicate what enzymatic processes are involved, under what conditions the amine-forming enzymes are produced by bacteria or what exact conditions are necessary for such enzymes to be active once formed. Attempts were made to produce histamine by washed suspensions but as the organisms were obtained from the surface of agar and buffered at pH 5.2, it is not surprising in view of more recent work (39) that the results were unsatisfactory,—as the organisms grown in this manner would contain little, if any, of the necessary histidine decarboxylase and also the pH of 5.2 is about 1 pH unit too alkaline for optimal activity. This will be dealt with below.

Virtanen *et al.* (93, 96) have shown the formation of  $\beta$ -alanine and  $\gamma$ -aminobutyric acid from aspartic and glutamic acids respectively by the root-nodule bacteria and have also shown the quantitative formation of cadaverine from lysine by *E. coli*. They have avoided the difficulty of interpreting experiments in which growth is occurring by using thick suspensions of the organism and incubating these in a non-nutrient medium containing phosphate and lysine only, estimating the cadaverine formed by isolation as the picrate.

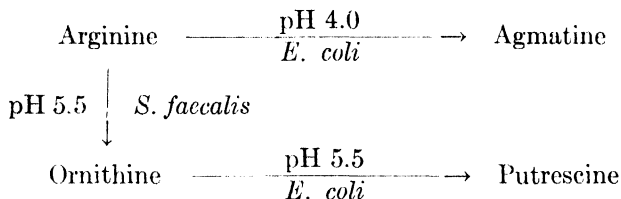
The first study of the enzymes involved in the formation of amines has been made by Gale (39) using washed suspensions of *E. coli* and investigating their power to decarboxylate amino acids by the Warburg manometric technique. Of 14 strains of *E. coli* investigated, 12 decarboxylated arginine to form agmatine; 12, histidine to histamine; 13, lysine to cadaverine; 12, ornithine to putrescine; and 9, glutamic acid to  $\gamma$ -aminobutyric acid. The decarboxylases involved are active over a restricted range of pH having optimal levels at the unusually acid values of 4.0 for arginine, histidine and glutamic acid, 4.5 for lysine and 5.0 for

ornithine. The enzymes proved very sensitive to temperatures above 20° to 25°, and washed suspensions grown at 27° proved markedly more active than those prepared from cultures grown at 37°. It is doubtful whether this thermolability is a property of the enzymes themselves or of the enzymes as they function in washed suspension, since the glutamic acid decarboxylase is far less thermolabile in *Proteus vulgaris* (42) and the histidine enzyme is formed better at 37° than at 27° in *Clostridium welchii* (42). At the optimum pH and at an experimental temperature of 30°, the washed suspensions of *E. coli* carry out a simple quantitative decarboxylation of the amino acids mentioned (without any coincident deamination) thus producing the corresponding amines which have been isolated and identified in each case.

The organisms contain only the decarboxylases if they are grown under certain specific conditions. The early work on growth experiments in which amines were produced had all been carried out in media containing fermentable carbohydrate or glycerol, so in this work the organisms were grown at first in 2% glucose broth without chalk so that the pH fell during incubation. Washed suspensions obtained from such cultures contained active amino acid decarboxylases of the type described above. By substitution of the glucose by other sugars, it was shown that the presence of glucose during growth has no specific effect but that any fermentable carbohydrate in the medium will give rise to a culture containing active decarboxylases. Further it was shown that the fermentable carbohydrate acts in this respect by acting as a source of acid and that the factor controlling the production of amino acid decarboxylases is the pH of the growth medium; bacteria grown at pH 5 in a non-carbohydrate medium having higher activities than those grown in a similar medium containing glucose. In all cases organisms grown at pH 7 in non-carbohydrate media have very little decarboxylase activity (hence the failure of Eggerth's experiment with washed suspensions (30)) and this activity is greatly increased (20 to 100-fold) by growing the organism at pH 5,—the lower the pH during growth, the greater the activity of the decarboxylases, within physiological limits.

The product obtained by the decarboxylation of arginine by

*E. coli* proved to be agmatine, whereas the amine previously reported as the product of bacterial putrefaction of arginine is putrescine (4, 10, 56). In those cases where mixed cultures have been used (4) it was suggested (39) that an organism was present which brought about the breakdown of arginine to ornithine before the decarboxylation occurred. Hills (53) has shown that many of the streptococci possess an enzyme capable of bringing about this reaction and Gale (41) has shown that *E. coli* and *Streptococcus faecalis*, when grown in symbiosis, attack arginine, according to the scheme:



Thus the two organisms grown in symbiosis attack arginine to produce either agmatine or putrescine according to the pH. The production of putrescine from arginine by *E. coli* alone has been reported (10, 56) but in these cases it is noticeable that the organism has been grown in a synthetic medium containing arginine as sole source of nitrogen so that, presumably, the organism has to elaborate some method of obtaining nitrogen from the amino acid for growth purposes before any decarboxylases are formed or can come into play.

The studies on decarboxylation by washed suspensions have been extended, up to the time of writing, to *Streptococcus faecalis* (40), *Proteus sp.* and *Clostridium spp.* (42). *S. faecalis* does not attack any of the amino acids decarboxylated by *E. coli* but washed suspensions grown in glucose broth decarboxylate tyrosine to tyramine at an optimum pH of 5.0, the enzyme responsible again being formed in response to acid growth conditions, particularly during the cofermentation of glucose. The enzyme is strictly specific as the washed suspensions decarboxylate no other amino acids tested (namely, phenylalanine, tryptophane, serine and alanine). Six out of seven strains tested possessed the

tyrosine-decarboxylase, the more saccharolytic strains having the greater activity. Of 16 coliform strains (39), 9 of the genus *Proteus* and 17 of the genus *Clostridium* (42) that were tested, none possessed the tyrosine-decarboxylase.

Of the 9 representatives of the genus *Proteus* (42) (which included *P. vulgaris*, *P. morganii*, the strains Kingsbury, HX2, HX19, etc.), 7 decarboxylated glutamic acid to  $\gamma$ -aminobutyric acid and 2 produced putrescine from arginine very slowly. The glutamic decarboxylase is similar in its properties to the enzyme in *E. coli* but is much less sensitive to temperature increase. It would seem that organisms of the genus *Proteus* cannot be considered as important amine producers.

Kendall and Schmidt (63) observed that when *Clostridium welchii* is grown in a carbohydrate medium, a non-specific toxic substance is produced in the medium which has the physiological reactions of histamine. Later (62) the substance was isolated from a bulk experiment and identified as histamine but apart from showing that this amine is produced only when the organisms are growing in the presence of carbohydrate, these workers did not obtain the detailed conditions for its production. Some 72 strains of *C. welchii* were investigated and histamine was found in the majority of cases. Eggerth (30) included 4 strains of *C. welchii* amongst the organisms he investigated and found all four to produce histamine when grown in the presence of salts, glucose, meat-extract, histidine etc. Gale (42) proceeded to investigate the power of washed suspensions of *C. welchii* to decarboxylate amino acids under the general conditions found satisfactory with other organisms (39, 41). Of 10 strains investigated, 9 decarboxylated histidine to histamine, and all decarboxylated glutamic acid. The latter decarboxylase again is similar in its properties to the enzyme in *E. coli* but the histidine-decarboxylase differs in several ways from the histidine enzyme in coliform organisms. In the first place, it has a pH optimum of between 2.5 and 3.0 compared with 4.0 in coliform bacteria. Why this should be is not clear. In the coliform organisms, the pH-activity curve for histidine-decarboxylase falls steeply on the acid side of 4.0 and the enzyme is almost inactive at pH 3.5;



and it has been suggested that this is due to denaturation of the enzyme protein, and, in actual practice, it is often found that the organisms coagulate below pH 4.0. This is not the case with *C. welchii* where coagulation of the organisms does not take place even at pH 1 and the pH-activity curve for the histidine-decarboxylase is approximately symmetrical. Thus the difference in the apparent properties of the two enzymes may be due to the greater resistance to denaturation of the proteins in *C. welchii* over those of coliform organisms. (See dotted curves, fig. 2.)

As a result of the lower pH optimum of the *C. welchii* histidine-decarboxylase, this enzyme requires more acid conditions for its formation than are necessary with *E. coli*. Thus in glucose broth, the histidine enzyme only appears late in the growth period when the general medium pH has fallen below 5. When the organism is grown in non-carbohydrate media, it is found as before that the lower the growth pH, the more active the decarboxylases, but in the case of the histidine enzyme the activity of a culture grown at pH 5 is  $Q_{CO_2} = 10$  compared with a figure of 60–70 for the same organism grown in the presence of glucose. It is not possible to obtain cultures from non-carbohydrate media at pH values much below 5 and it appears that, in the case of this organism, histamine is only produced rapidly *in vivo* during the cofermentation of carbohydrate as it is only by this means that a pH sufficiently low for the formation of the enzyme during growth can be obtained. It is probable that a very saccharolytic organism like *C. welchii* may, during the fermentation of sugar, especially when in a heterogeneous medium such as an infected muscle, produce a pH in its immediate vicinity below that of the medium in general. Washed suspensions of *C. welchii* are, of course, fully active in the absence of sugar, but it is unlikely that much histamine will be formed in infective conditions unless the organism has the opportunity to ferment carbohydrate. This is of importance in gas gangrene as *C. welchii* ferments muscle glycogen with the production of acid and gas, and washed suspensions obtained from cultures grown in the presence of glycogen are active histamine-producers. The fermentation of glycogen is strictly adaptive.

Table 2 shows the distribution of the amino acid decarboxylases

in organisms that have been investigated so far by the washed suspension technique. In each case a representative organism has been chosen; but it has been shown that there are marked strain differences, as, for example, a representative *E. coli* possesses decarboxylases for arginine, ornithine, lysine, histidine and glutamic acid but some strains of coliform organisms possess only one or two of these enzymes when grown under the appropriate conditions. In all the work discussed, values of  $Q_{CO_2}$  ( $= \mu\text{l. CO}_2$  liberated from the specific substrate per hour per mg. dry weight of organism) less than 1 have been regarded as insignificant so that certain enzymes of very weak activity may have

TABLE 2  
*Distribution of amino acid decarboxylases*

ORGANISM	ENZYME-SUBSTRATE					
	Arginine	Ornithine	Histidine	Lysine	Tyrosine	Glutamic acid
<i>Escherichia coli</i> .....	+	+	+	+	—	+
<i>Klebsiella pneumoniae</i> .....	—	+	+	+	—	—
<i>Streptococcus faecalis</i> .....	—	—	—	—	+	—
<i>Proteus vulgaris</i> .....	—	+	—	—	—	+
<i>Proteus morganii</i> .....	—	+	—	—	—	+
<i>Clostridium welchii</i> .....	—	—	+	—	—	+
<i>Clostridium sporogenes</i> .....	—	—	—	—	—	—
<i>Clostridium septicum</i> .....	—	+	—	—	—	—
<i>Clostridium aerofetidum</i> .....	—	—	—	—	+	+

Presence of activity is indicated by +.

been disregarded, when their presence might show up in prolonged growth experiments. Thus strains of *E. coli* and *Proteus vulgaris* have been reported to produce tyramine from tyrosine (82) and isoamylamine from leucine (11) whereas no sign of such activity was found in work with washed suspensions of these organisms (39, 42). It is also possible, of course, that these enzymes are confined to strains which were not represented in the work with washed suspensions. Thus the enzymes shown in table 2 cannot be taken to represent the maximum decarboxylating powers of the organisms concerned.

The distribution of the decarboxylases indicates that each enzyme is specific for the decarboxylation of one amino acid and

probably (39, 41) for the amino acid molecule itself. A surprising thing is the frequency of the occurrence of the glutamic acid decarboxylase which has been found in the majority of strains of *E. coli*, *Proteus spp.*, *C. welchii* and *C. aerofetidum* investigated; moreover it is usually present in a very active state (42), and it would seem that the metabolism of  $\gamma$ -aminobutyric acid in bacteria should repay study. The enzymes are not produced if growth occurs in an aminoacid-free medium (39, 41).

#### *Factors influencing bacterial decarboxylation*

Gale (39, 41, 42) showed in the course of the above investigations that (a) the decarboxylase activity of a washed suspension towards any particular amino acid varies with the "age of the culture" from which the washed suspension is prepared. Thus young cultures have little activity, the activity increasing during active cell division and reaching a maximum as growth ceases. The case of the histidine-decarboxylase of *C. welchii* differs from the usual type because, when growing in the presence of carbohydrate, the enzyme does not appear until late in the growth period when the pH of the medium has fallen below 5 (42). (b) The presence of oxygen during the decarboxylation by washed suspensions has no effect on the reaction, with the exception of the glutamic acid decarboxylase of *C. welchii* which is partially inhibited by the presence of oxygen (42). (c) There is evidence that the decarboxylases require a coenzyme or coenzymes for their action (39) but these have not been identified although they cannot be replaced by aneurin diphosphate (cocarboxylase).

The effect of growth conditions on the production of the enzymes has been discussed above. In every case studied, the decarboxylases are formed in response to acid growth conditions, the lower the pH during growth, the more active the washed suspensions obtained. Since the optimum pH of these enzymes is below 5.5 in every case and below 4.5 in most cases, it is probable that the amino acid can only be attacked when it possesses an undissociated carboxyl group. As the pH of the growth medium is lowered, the proportion of the amino acids in the undissociated acid state will increase and thus the response of the organism in producing more enzyme may be a type of adaptation. Pointing

out that decarboxylation of amino acids results in the formation of alkaline material, Hanke and Koessler (47) wrote "the production of amines from amino-acids seems to be a protective mecha-

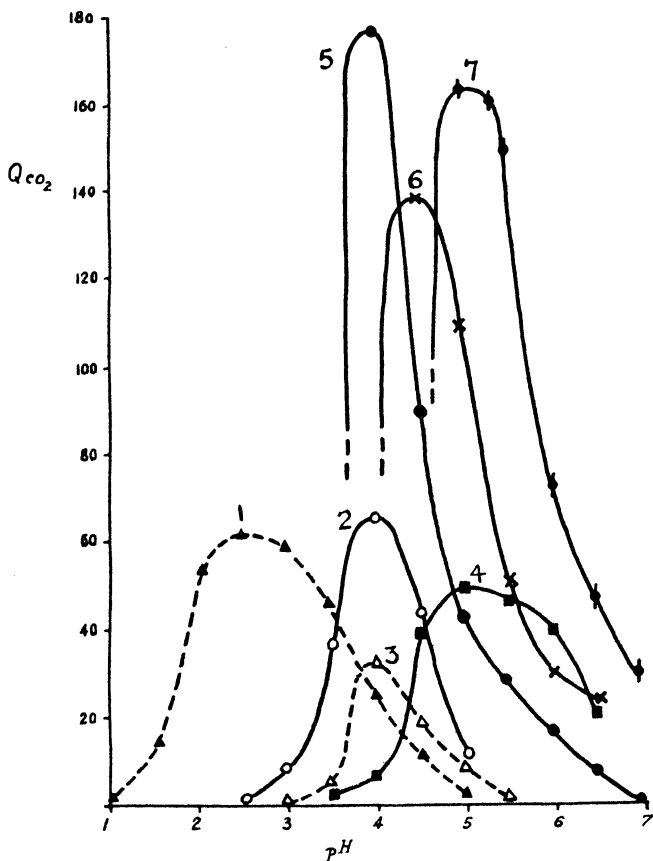


FIG. 2. VARIATION WITH pH OF THE ACTIVITY OF VARIOUS AMINO ACID DECARBOXYLASES IN WASHED SUSPENSIONS OF BACTERIA

1. Histidine-decarboxylase of *Clostridium welchii* (42). 2. Glutamic acid-decarboxylase of *Escherichia coli* (39). 3. Histidine-decarboxylase of *E. coli* (39). 4. Ornithine-decarboxylase of *E. coli* (39). 5. Arginine-decarboxylase of *E. coli* (39). 6. Lysine-decarboxylase of *E. coli* (39). 7. Tyrosine-decarboxylase of *Streptococcus faecalis* (40).

nism and is resorted to when the accumulation of H ions within the organism's protoplasm is incompatible with its normal life processes. The amines can thus be thought of as reaction

buffers." From a teleological point of view, it is difficult to assign any function to the amino acid decarboxylases at present although it must be remembered that when the reaction becomes acid, amino acids can no longer be attacked by deamination for the deaminases are inactivated and, at pH values lower than 5, carbohydrate itself is but slowly attacked, and the production of decarboxylases may be the method by which the organism extends its range of existence, utilizing amino acid decarboxylation when other substrates and methods of attack are no longer available. A further suggestion is that the decarboxylases may serve the purpose of providing carbon dioxide, which is essential for the growth of many bacteria (45), under conditions too acid for sufficient gas to be retained in normal solution in the medium.

### 3. DEAMINATION ACCOMPANIED BY DECARBOXYLATION

Figure 3 shows the variation with pH of the activity of glutamic acid deaminase (87) and glutamic acid decarboxylase (39) of *E. coli*. It can be seen that not only are the optimum pH values for these two enzymes of the same organism widely separated and on opposite sides of neutrality but that neither enzyme is at all active when the other is optimally active. Further, the decarboxylase is active over such a restricted range that there is no pH at which both enzymes are effectively active together. It is not possible to say whether this case, the only one for which complete data are available at present, is representative of deaminases and decarboxylases in general, although it is significant that all the deaminases so far studied are optimally active at pH 7.5 to 8.0 while all the decarboxylases have optimal pH values at or below 5.0. This may mean that for decarboxylation to occur, the  $\text{—COOH}$  group must be undissociated and, for deamination, the  $\text{—NH}_3^+$  group. Hence it is probable that bacteria cannot attack amino acids by both deamination and decarboxylation *simultaneously*. Thus there is no record of alcohols (which would be formed by coincident decarboxylation and hydrolytic deamination) being produced from amino acids by bacteria, although a series of papers by Ehrlich between 1905 and 1912 has shown the production of such by yeast in culture. In culture experiments which last over several days and in which

considerable changes of pH may occur due to the metabolic activities of the growing organisms, products of deamination and decarboxylation might be expected as a result of the two processes occurring at separate times during the incubation. Thus Brasch (19, 20) has reported the isolation of propionic acid from a culture of *Clostridium putrificum* growing in a medium containing aspartic acid: similarly he has obtained butyric acid from glutamic acid. It should be of interest to investigate the action of washed suspensions of yeast upon amino acids at various pH

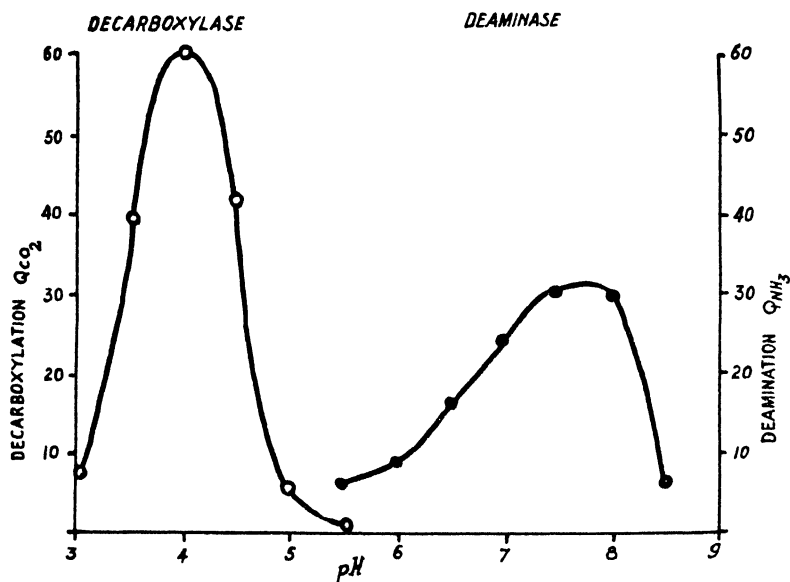


FIG. 3. VARIATION WITH pH OF THE ACTIVITIES OF THE ENZYMES OF *ESCHERICHIA COLI* WHICH ATTACK *L*-GLUTAMIC ACID (39, 87)

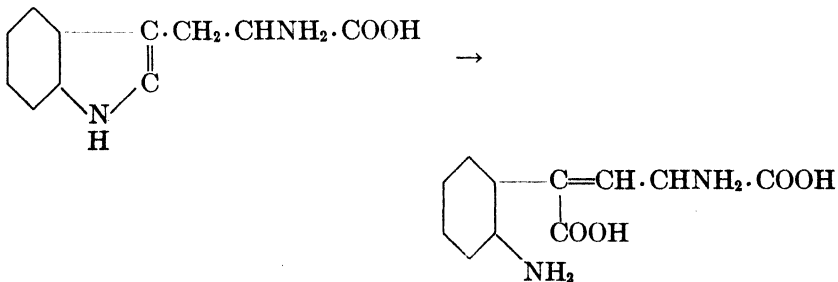
values, to determine whether the results obtained by Ehrlich are due to simultaneous deamination and decarboxylation by enzymes of different properties from those found in bacteria, or whether the alcohols are produced in two stages as the growth medium reaction alters.

#### 4. SPLITTING OF THE AMINO ACID MOLECULE

Few cases of such a reaction by bacterial enzymes have been reported up to the present. Ackermann (2) showed that mixed

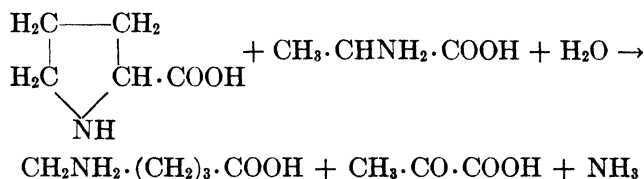
cultures of organisms will produce ornithine when growing in a medium containing arginine. Hills (53) has shown that this reaction is carried out by certain streptococci with the production of ammonium carbonate in addition to ornithine. He was unable to show the intermediate formation of citrulline, urea, etc., and the precise mechanism of the reaction is still obscure. The same change is carried out by some strains of *Lactobacillus acidophilus* in washed suspension (40) the optimum pH for the reaction being around 6.0. Again urea is not an intermediate substance in the removal of the guanidine nucleus. Ackermann (6) has also shown the formation of citrulline from arginine by mixed cultures and Horn (59) has obtained this reaction by growing *Pseudomonas aeruginosa* in a determined medium containing arginine as sole source of nitrogen. The yield was small.

It has been mentioned above that no attempt to identify an intermediate substance in the production of indole from tryptophane has met with success and it may be that this is also a case in which the amino acid molecule is split (into indole and alanine?) as a preliminary to further breakdown. Such a breakdown consisting of "reductive fission" of the tryptophane molecule has been postulated by Baker and Happold (13) although there is no experimental evidence in support of such a change as yet. A further type of attack on the tryptophane molecule that might be classed under this heading has been shown by Kotake (67): *Bacillus subtilis* produces kynurenic acid, kynurenine and anthranilic acid from tryptophane in the presence of glycerol and aluminum phosphate. It is suggested that the first step in this breakdown is the production of kynurenine from tryptophane:

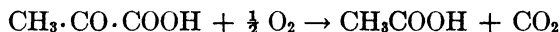


followed by disruption of the kynurenine along two paths to kynurenic acid and anthranilic acid.

Stickland (89) has also shown that when *l*-proline is reduced by *l*-alanine in the presence of washed suspensions of *C. sporogenes*, the ring of the proline molecule is split to produce  $\delta$ -amino-valeric acid:



after which occurs the second step:



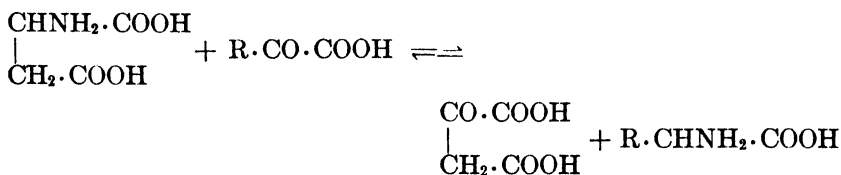
#### ANABOLIC CHANGES INVOLVING AMINO ACIDS

While our knowledge of the catabolic changes involving amino acids is considerable, as this review has shown, and is reaching a stage when the enzymes concerned can be studied with a fair degree of exactness, this cannot be said to be the case with anabolic changes. Leaving aside the discussion of growth factors, it is true to say that some organisms such as certain strains of *E. coli* can grow in a non-amino-acid synthetic medium containing ammonia whilst others such as *Staphylococcus aureus* cannot. In the former case the organism must be able to synthesize the amino acids of its protoplasm from carbon compounds and ammonia. In many such cases the addition of amino acids hastens the rate of growth and it is generally assumed that this is due to the fact that certain of the amino acids can only be synthesized slowly by the organism so that the rate of synthesis restricts the rate of growth, with the consequence that addition of these amino acids speeds up the growth. When such an amino acid is added to the simple growth medium, it is not clear whether it is first deaminated and then rebuilt from its breakdown products or whether it is directly assimilated by the organism without such intermediate breakdown. It has been shown (38, 43) that some of the deaminases, at least, are almost



inactive in very young cultures and this may be a factor contributing to the economy of the cell during its actively anabolic stage. In those cases where the organism cannot grow on a non-amino-acid medium, this is presumably due to its inability to synthesize some "essential" amino acid from simple substances. Thus some strains of *Eberthella typhosa* and *S. aureus* cannot grow in a simple medium unless tryptophane is added (36). Fildes (34) suggests that the loss of such a synthetic function is brought about by continued growth of the organism in such an environment that the essential factor is supplied and the need to synthesize it does not arise. In this connection, Fildes and his co-workers (36) showed that a strain of *E. typhosa* which would not grow in a non-amino-acid medium without the addition of tryptophane, could be "trained" to dispense with the tryptophane by continued subcultivation into media containing progressively smaller and smaller amounts of the amino acid. If Fildes' (34) theory is correct it should be possible to demonstrate the reverse of this training process: the loss of synthetic power by an organism growing in the presence of the synthesized material.

It is generally assumed that synthesis of amino acids is accomplished by reversal of the enzymes involved in amino acid breakdown. Of all such enzymes studied, two have so far been shown to possess the property of reversal: the *l*-glutamic acid dehydrogenase studied by Adler *et al.* (9) and the aspartase studied by Quastel and Woolf (78). In animal tissues, Braunstein and Kritzmann (21, 22) have demonstrated a transamination whereby the amino group of either of the natural dicarboxylic amino acids can be transferred to a monocarboxylic keto acid with the formation of the dicarboxylic keto acid and a monocarboxylic amino acid:



Thus, once either of the dicarboxylic amino acids has been synthesized, then the way is open to the formation of other amino

acids once their corresponding keto acids have also been synthesized. The transamination has yet to be demonstrated in bacteria.

A further method for the synthesis of aspartic acid has been shown by Virtanen and Laine (94, 95) for the symbiotic genus *Rhizobium*. In this case, it is claimed that the organism fixes atmospheric nitrogen with the formation of hydroxylamine which then combines with oxaloacetic acid, produced by the host-plant from carbohydrate, to form aspartic acid:



Virtanen and Laine (94) claim to have further demonstrated the transfer of the amino group of the aspartic acid to pyruvic acid to form alanine in the presence of the host-pea.

#### SUMMARY

An account is given of our knowledge of bacterial metabolism of amino acids with especial emphasis laid on the enzymes involved in the primary breakdown of the amino acid molecule. Bacterial enzymes can attack amino acids by (a) removal of the  $\alpha$ -amino-group (b) removal of the terminal carboxyl group (c) by combined deamination and decarboxylation or (d) splitting the molecule into simpler molecules. The third alternative probably does not occur in practice.

Amino acids may be deaminated in a number of ways. Experiments with washed suspensions of bacteria and with cell-free enzymes isolated therefrom show that the process can be carried out by (a) oxidation or dehydrogenation (b) desaturation (c) hydrolysis (d) reduction of the amino acid molecule in specific cases. Also certain strict anaerobes carry out a process of mutual oxidation-reduction between pairs of amino acids that results in their deamination. The deaminases all appear to be optimally active between pH 7.5-8.5. The deaminase activity of bacterial cells varies with the age of the culture from which the cells are taken, the composition of the growth medium, and the degree of anaerobiosis during growth. Co-deaminases have been described in certain cases.

Amino acids may be decarboxylated to give amines under acid

conditions, the specific decarboxylases being optimally active at or below pH 5.0. Such decarboxylases are only formed when the organisms are grown under acid conditions and the decarboxylase activities of the cells vary under varying conditions of growth.

A few isolated cases have been described of amino acids being split into simpler molecules before undergoing further degradation.

Little is known of the way in which bacteria synthesize amino acids although the deaminases of the two dicarboxylic amino acids are known to be reversible and it is possible that the synthesis is accomplished by the production of aspartic and glutamic acids followed by transfer of the amino group from these to keto acids with the formation of other amino acids.

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## CORYNEBACTERIUM DIPHTHERIAE

### A CORRELATION OF RECORDED VARIATIONS WITHIN THE SPECIES<sup>1</sup>

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Diphtheria is one of the diseases either diagnosed or confirmed in the laboratory by the isolation and identification of the causative microorganism, therefore it is of great practical as well as theoretical importance that we know as much as possible concerning the range of variation and the dissociative behavior of the causative organism commonly referred to as "the diphtheria bacillus." The organism has been studied extensively, but often the investigations were confined to certain aspects while totally ignoring other aspects or even previous studies along the same lines. It is not surprising then to find in the literature numerous conflicting reports on the behavior of the diphtheria bacillus.

Our present pleomorphic concept of bacterial species is one which depicts order and not chaos within each species. For several bacterial species it has become possible to correlate many of the chemical and physical properties of the microorganisms with colony form. A preliminary review of the literature (Morton, 1935b) suggested that this is also possible in the case of the diphtherial species. It is from this point of view that the experimental work reported elsewhere (Morton, 1940) was carried forward and the following review prepared.

So-called dissociative studies of recent times have contributed to a better understanding of a bacterial species; and the variations already reported fall into a generally consistent pattern. In the case of the diphtheria bacillus, a consideration of the earlier

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reports of its variation shows that, on the whole, the isolated observations do not represent incongruities within the species but rather an orderly trend in the variation process. Many of the seemingly independent variations can now be correlated with variations in colony form. So evident is this that it is often possible to predict many of the properties of a culture by merely establishing the colony form to which it belongs. Despite the relatively short period in which the various culture phases have been recognized as such, there is present in the older literature much valuable information which, if rightly interpreted, contributes to our better understanding of the variation problem.

The review which follows endeavors to treat only of the nature and degree of the recorded variations of the diphtheria bacillus and the extent to which they may be correlated with the recognized colony forms. The characteristics or properties are considered in the following order: (1) Colony forms; (2) Appearance of growth in liquid media; (3) Stability of the cell suspensions in salt solution; (4) Virulence; (5) Toxigenicity; (6) Serological reactions; (7) Hemolysis; (8) Fermentation reactions; (9) Chromogenesis; (10) Reduction of potassium tellurite; (11) Cell morphology.

## 1. COLONY FORMS

*The smooth (S), intermediate (SR), and rough (R) colony forms*

Six years after Loeffler's original description of the colonies produced by the diphtheria bacillus, variations were reported by Klein (1890). Peters (1897) also noticed a variation in the colonies, but the first to give serious attention to colony variation of the diphtheria bacillus were Cobbett and Phillips (1897). In addition to describing "shiny" and "matt" colonies, Zupnik (1897) confirmed the observations of Cobbett and Phillips that the composition of the medium is very important in determining the colony form. Serum or glycerol agar do not differentiate the shiny and matt colonies, but the difference can be observed on ordinary agar. These findings were confirmed by Schick and Ersettig (1903); and numerous others have reported the occurrence of two distinct colony forms for the diphtheria bacillus.

Following Arkwright's (1921) coinage of the terms *smooth* and *rough* to describe the forms of the colonies produced by the intestinal group of microörganisms, and the application of these terms to the colony forms of other species, Scott (1923) named the two kinds of colonies of the diphtheria bacillus as smooth and rough. Cowan (1927) has claimed a separation of the Park No. 8 and of another strain into smooth and rough colonies by selective cultivation; however, one wonders if she had the pure S form, because her smooth colonies are described as "less raised and less dense" than the R colonies. The purity of her R form is also open to question, for it is stated to produce a smaller pellicle than the S. A very important observation by Miss Cowan is that in the development of the R form the organisms are seen to pass through a stage in which the colonies are, for the most part, very large, irregular and coarsely granular, and the individual bacilli are also larger and of a great variety of shapes, often showing branched forms. Parker (1928) mentions smooth and rough strains, but here again the findings are not in accord with what one usually thinks of as the actual smooth and rough colony forms. Okell (1930) was not able to confirm Cowan's work even with the same cultures used by the latter; but this is not at all unusual since cultures very often cannot be dissociated at will. Yü (1930) was able to dissociate the S colony form to what he regarded as the R but was unable to change the R back to the S. Viewing the pictures of his colonies, however, one wonders if he had the actual R form.

Study of the colony form of the diphtheria bacillus assumed a different aspect following the description by Anderson, Happold, McLeod, and Thomson (1931) of "gravis" and "mitis" forms. After routine laboratory examinations over a period of eight years, they concluded that there are two distinct, stable types of the diphtheria bacillus with which are associated the following characteristics.

Type *gravis*. The growth on potassium tellurite chocolate medium after 48 hours is gray or gray-black. The colony is like that of *C. xerosis*, but heavier. The cell morphology on media, other than Loeffler's, is that of a very short diphtheroid, usually without granules. On

Loeffler's medium the granules are sometimes well marked and at other times scanty. There is a pellicle and granular deposit in broth; and the organisms give rise to unstable suspensions in saline, are non-hemolytic, possess typical fermentation reactions (no acid with sucrose, acid with glucose, maltose, galactose and invariably with dextrin, starch and glycogen), and grow vigorously on the special chocolate tellurite medium. The organisms are associated with severe cases of diphtheria.

*Type mitis.* The growth on the potassium tellurite chocolate medium after 48 hours is black. The colony is like that of *C. hofmanni*, but finer and more translucent. The cell morphology is that of the textbook *C. diphtheriae*, the bacilli usually being long and the granules well marked. There is uniform turbidity in broth; and the organisms produce stable suspensions in saline, are hemolytic, give recognized fermentation reactions, (no acid with starch or glycogen and inconsistent results with dextrin), and are partially inhibited upon subculturing to the chocolate tellurite medium. The organisms are associated with mild cases of diphtheria. The type *mitis* is commonly non-virulent for guinea pigs, but the type *gravis* almost always shows some degree of virulence.

Later (1933), Anderson and his colleagues proposed a third, type *intermedius*, that was said to be associated with diphtherias of intermediate severity. Its description follows.

*Type intermedius.* The type of growth on potassium tellurite chocolate agar, serum agar, heated blood agar, or plain agar is fine. The colonies are flat with central knob and slightly crenated periphery. On potassium tellurite chocolate agar the colonies are very fine with slightly raised black center and thinner translucent periphery. There is granular growth in broth, which settles rapidly. Neither starch nor glycogen is fermented. The morphology is that of a barred diphtheroid with metachromatic granules variable but often poorly developed.

There are at least two serious faults in the above descriptions and assumptions. First, not much information is conveyed in a description which states that a colony form is like that of *C. xerosis* or of *C. hofmanni*, as bacteriologists have realized since the works of Arkwright (1921) and Hadley (1927, 1937) demonstrated that no microorganism has a single and distinctive colony

form. The color plates, however, convey a fairly good picture of the two colony forms, which Anderson, *et al.*, were attempting to describe. Second, it is highly questionable whether or not it is possible to associate slight variations in clinical manifestations with colony form. Menton (1932) and others believe that the terms "gravis" and "mitis" have no biological basis. Although Anderson, Cooper, Happold, and McLeod (1933), and Carter (1933) claim that the colony forms and fermentation reactions are stable over a long period of cultivation, such claims are not in strict agreement with our present conceptions of bacterial variation. It is not surprising then that other workers are in disagreement, notably Wright and Rankin (1932), and Menton, Cooper, Duke, and Fussell (1933). Evidence of variation of the types *mitis* and *gravis* is given by Christison (1933) who observed a type *mitis* strain change its manner of growth in broth from turbidity and compact sediment to that of a pellicle and granular sediment with the supernatant fluid remaining clear, by subculturing in broth of pH 7.8 at three-day intervals. In addition, Menton was able to transform the type *gravis* colony to the type *mitis* form by the addition of commercial antitoxin to the medium. The work of Robinson (1934) shows that the various type strains of *C. diphtheriae* are relatively stable in ordinary stock cultures, but that by the usual *in vitro* and *in vivo* methods employed to promote variation, these strains show marked variation in most of the type characteristics. These latter findings are in keeping with our concepts of the dissociative behavior of a bacterial species. Menton (1932), Murray (1935a), and others have observed that typical colonies of one form may have the biochemical characteristics of another colony form; and numerous workers, in addition to Robinson and Peeney (1936), have contributed evidence for the variations in type characteristics of the strains.

Not only is type *gravis* not always associated with severe cases of diphtheria, nor type *mitis* with mild cases, but occasionally both types are isolated from patients or found in stock cultures. Parish, Whatley and O'Brien (1932) found that the mortality rate in human beings is about the same for organisms of both

colony forms and that, over a large region, the type *gravis* strains have been reported to be as prevalent in mild cases as in severe cases. Although the range in virulence of type *mitis* strains for guinea pigs was found by Anderson, *et al.*, to be greater than that of type *gravis* strains, the latter showing more uniform virulence, only three of the 90 strains tested for virulence by these workers, (employing the intracutaneous method on guinea pigs), gave a very marked reaction and these were type *mitis* strains. Whatever the type, 50 per cent or more, of the strains produced a moderate reaction in the guinea pigs. Bearing in mind the work of Powell (1923), who found that 40,000 to 400,000 diphtheria bacilli are required for a minimal reacting dose in the intradermal method of estimating virulence, one may well hesitate to interpret a reaction to the test dose as doubtful, slight, moderate, marked or very marked, unless the dose is more sharply standardized. If administered either subcutaneously or intradermally into guinea pigs, the type *mitis* strains are as virulent as the type *gravis* strains (Parish, Whatley, and O'Brien). When administered either intravenously or subcutaneously into rabbits, the type *mitis* strains appear to be more virulent.

It is quite obvious from the data that no one particular colony form exemplifies the diphtherial species. Diphtheria bacilli are either virulent or nonvirulent, and, if virulent, this quality is not necessarily associated with any particular colony form as, for example, is the case with the pneumococcus. The quantitative differences between virulent strains of diphtheria bacilli can be explained on the basis of S-R variation.

Later and more detailed reports of rough colonies of the diphtheria bacillus are those by Whitley (1934), who found a "rough" form present in nose and throat cultures; by Morton (1935a, 1940) and Hobby (1935), who produced R forms by forced dissociation; and by Bisset (1938) who encountered R colonies in strains which were being repeatedly subcultured. Thus far, of all the descriptions of the rough colony forms, those by Morton and by Hobby appear to represent the ultimate rough form, in that they more nearly fulfill the criteria for a rough culture phase as described by Hadley. Following are the criteria for a rough,

or R, colony form, in general. The margin is very irregular; the surface is very flat, uneven and granular; the organisms grow as a granular sediment in broth and clump spontaneously in physiological salt solution. Evidence for this type of colony does not appear very often in the literature, in spite of the fact that workers have designated some of their colony forms as rough.

The usual criteria for the smooth, or S, colony form, in general, are: the margin is round and even; the surface is convex and smooth; the organisms grow in broth with a uniform turbidity, and produce a stable suspension in physiological salt solution. Workers prior to Baerthlein and Arkwright did not always search for these cardinal points, but from their meager descriptions one is able to recognize some of the colonies, undoubtedly, as of the smooth form. The first impression one receives from the description by Anderson, Happold, McLeod, and Thomson (1931) is that their type *mitis* colonies are the smooth form. This impression is verified when one examines the colonies by some special means of illumination, as did Murray (1935b).

During the transformation of the smooth to the rough colony form, the surface of the colonies becomes flattened, irregular and granular; and, as Cowan mentioned, the colonies also become larger. It is this intermediate form, SR (somewhat removed from the pure S), which produces the pellicle type of growth with sediment and clear supernatant, so often obtained with diphtherial cultures. One immediately recognizes the similarity of the type *gravis* colonies described by the English workers and the SR colony form.

From the description by Anderson, *et al.*, it appears that some of their intermediate forms, which could not be classified as either type *mitis* or type *gravis* colonies, represent, perhaps, a stage of the diphtherial organism somewhat removed from the SR stage and approaching roughness, whereas other forms in this group are suggestive of a dwarfed smooth colony. The term "intermediate", when used in this connection does not necessarily mean that such a colony is intermediate between those of types *mitis* and *gravis*. In the terminology employed in respect to smooth and rough colonies, the term "intermediate" signifies



that the colony has an appearance that is in between those of the smooth and the rough forms; the "intermediate" colony can be observed to arise from one form and proceed into the other.

When one reads the reports of the incidence of types *gravis* and *mitis*, and intermediate forms in various localities and epidemics, it is readily apparent that the predominating type is likely to vary from one locality to another and from one epidemic to another. If virulence is not necessarily associated with one particular colony form of the diphtherial species, as numerous workers have shown, then it is possible for one colony form to predominate in a given epidemic or in a certain locality and to be replaced by another colony form during another epidemic or in another locality, which, in fact, is substantiated by the findings of Frobisher (1939).

In no other species of microorganisms have colony forms been named according to the quantitative variations in clinical manifestations which they produce, and, from the number of discordant reports, this does not appear to be the most satisfactory basis for nomenclature for the diphtherial species. The inadequacy of the classification is exemplified by the necessity for the creation of sub-groups, as was done by Wright, Christison, Rankin, Pearson, and Cuthbert (1935), and Stuart (1938), who found no correlation between virulence, colony form, and the ability of a strain to ferment starch. It is surprising that earlier students of types *gravis* and *mitis* did not attempt to associate the various colony forms with S-R variation, a system of variation and nomenclature which has been found applicable and useful for hundreds of other bacterial species.

#### *Dwarf colony form (D)*

It is interesting that in the same year in which colonial variation of the diphtheria bacillus first received serious study, dwarf colony forms were encountered. It was only the larger forms, however, the smooth and rough colonies, which were studied in the years that followed. Cobbett and Phillips in their studies in 1897 described extremely minute colonies of the diphtheria bacillus. When 10 cultures of virulent diphtheria bacilli were

grown on gelatin, 6 of them yielded two varieties of colonies, designated as large and small colony types. Two cultures yielded only the small colony type and two the large type only. When separated into pure cultures, the large and small colony forms maintained for many generations their characteristic difference in size. From pure cultures of the large colonies, all the colonies in subsequent generations were of the same large type. Pure cultures of the small colonies, however, although continuing to produce small colonies for many generations, occasionally gave rise to colonies of the large type. One strain maintained constantly the property of producing only the small colony form. Cobbett and Phillips at first were inclined to ascribe this phenomenon to their failure to separate completely the two varieties and not to a tendency to variation; but later they ruled out the possibility of impure cultures. The appearance of the two colony forms on gelatin was the only instance in which the strains varied, the strains were alike in virulence, microscopic appearance, and in their manner of growth in broth and upon media other than gelatin. These small colonies were extremely minute, often scarcely visible, and seldom as large as a half-millimeter in diameter.

According to the type of growth on trypsin-serum agar, Parker (1928) temporarily classified diphtherial cultures into heavy and light growers. The heavy growers were further classified into R-heavy and S-heavy growers. The light growers were not as easily differentiated from the heavy growers on serum agar in which the serum had not been trypsinized nor on plain agar. The light growers were in every case (16 strains) toxigenic. These light growers described by Parker, appear similar to the small colony forms described by Cobbett and Phillips, and may well be the forms which at the present time are referred to as dwarf (D) forms.

The "intermediate" type (of Anderson *et al.*) when plated from broth cultures was observed by Christison (1933) to yield frequently two kinds of colonies. One kind was irregular, granular, and flat with a central knob, which is suggestive of an sR colony form, and the other was much darker, glistening and

entire, but smaller and more convex than type *mitis* colonies of the same age. Since Christison made no statement as to the exact size of the colonies, it is difficult to interpret this colony form, but there is a possibility that it might be the dwarf colony form of the diphtheria bacillus.

Bacilli from type *gravis* colonies have also been observed to give off small-colony variants. Erzin (1936) describes two forms of growth for this type: typical type *gravis* colonies and "zarte" colonies. On blood agar the "zarte" colonies are round, convex, small, and hemolytic. On Clauberg's medium the colonies are soft, delicate, and tiny. On the medium of Gundel and Teitz the colonies are very small, delicate, appearing scarcely visible through a magnifying glass, and sometimes convex and shiny. On Loeffler's medium and in broth the organisms produce "typical growth for diphtheria." Upon the same five media, the type *gravis* or "typical" colonies produce "typical growth." Incubation of a broth culture of the "zarte" form at 37°C. gives rise after 10 days to a mixture of "zarte" and type *gravis* colonies. Incubation of a broth culture of type *gravis* at 37°C. gives rise after 34 days to a mixture of the two forms. The "zarte" form produces toxin but is less virulent for guinea pigs than type *gravis*.

Morton (1935a) reports small colony variants of the diphtheria bacillus, which are designated dwarf or "D" colonies. The small colony variants were produced by the aging of broth cultures of a Park No. 8 strain. The organisms are pathogenic for guinea pigs, are toxigenic, and ferment glucose and dextrin but not sucrose. They are agglutinated by immune serum prepared by immunizing rabbits against the parent culture of the Park No. 8 strain. In two filtration experiments, the organisms were not found to pass through a Berkefeld N filter. Hobby, in 1935, reported that her R type colony from a recently isolated diphtherial strain (RB-2T) either remained as such or was transformed to a minute or "G" colony.

Two forms of small colonies were observed by Mittag (1937) in addition to the commoner large forms. The small, irregular colonies, ("kleine, zarte, gekerbte Kolonien") appear to be the sR form, and the small, delicate, round colonies ("kleine, zarte,

runde Kolonien") appear to be the dwarf colonies. The organisms were pathogenic for guinea pigs.

Our review shows that small colony forms have been described for the diphtheria bacillus on at least six different occasions. The ability to produce an extremely small colony form, in addition to the more common large colony forms, is not a unique characteristic of the diphtherial species but seems to be a property inherent in many bacterial species. Similar forms have been described for the following: *Bacillus megatherium* (Rettger and Gillespie, 1933), *B. mesentericus* and *B. vulgatus* (Flynn and Rettger, 1934), *Clostridium welchii* (Roe, 1932, 1934), *Diplococcus pneumoniae* (Dawson, 1928; Eaton, 1934), *Eberthella typhosa* (Fromme, 1911; Eisenberg, 1914), *Hemophilus pertussis* (Kimball, unpublished data), *Lactobacillus acidophilus* (Kopeloff, 1934), *Neisseria gonorrhoeae* (Raven, 1934), *Pseudomonas fluorescens* and *Ps. pyocyanea* (Eisenberg, 1914), *Salmonella aertrycke* and *S. schottmuelleri* (Fürth, 1922; Koser, 1930), *S. suispestifer* (Orcutt, 1923), *Shigella equirulis* (Edwards, 1931), *S. dysenteriae* Shiga (Arkwright, 1921), *S. sonne* (Koser and Styron, 1930; Koser and Dienst, 1934; Chinn, 1936), *Staphylococcus aureus* (Swingle, 1934) and a group C hemolytic streptococcus (Morton and Sommer, unpublished data). In many of these cases the small colony forms were called dwarfs, and in other cases they were called "G" colonies in spite of the fact that they did not satisfy the criteria for the G type colony. If more care had been exercised in the classification of small colony forms, less confusion would now exist concerning the G forms. These dwarf forms are not at all unusual. One notices that they may occur under normal conditions of cultivation, but that their ratio to the larger colony forms is quite small. Frequently the dwarf colony forms are produced under the influence of aging or by growth in the presence of lithium chloride. One also recognizes that they are produced from cultures that are showing some evidence of, or a tendency toward, the rough phase. When practically the same phenomenon has been observed and described for at least 20 bacterial species, representing more than 12 different genera, it would seem to warrant recognition as a definite phase in bacterial variation.

*Gonidial colony form (G)*

In addition to the small colony forms mentioned above, which have been described by various workers since 1897, a few of the observations warrant special attention, because a small colony (G) form was obtained under special conditions of cultivation, and because it represents growth from *filterable* elements of the bacteria. In this respect the G colony form is unique. Hauduroy (1927) obtains such colonies from filtrates of diphtherial cultures by serially culturing the filtrates on solid media. This is accomplished by seeding a few drops of the filtrate on an agar plate. After suitable incubation the surface of the plate is washed with a few drops of sterile broth, and the washings are transferred to another fresh, sterile plate, this process being carried forward in series. Smith and Jordan (1930) describe growths from 11 of 19 filtrates of aged diphtherial cultures after serial transfers of the filtrates as recommended by Hauduroy. Hadley and Richardson (Hadley, Delves and Klimek, 1931) and Morton (1932) also report small colonies (designated as G colonies) from filtrates of diphtherial cultures.

Since many workers have been erroneously calling all small colonies G colonies through failure to establish that the organisms therefrom fulfill the necessary requirements, these criteria as proposed by Hadley (1931) will be reiterated. They are as follows:

"1. Visible signs of culture development appear in the filtrate, or in subcultures from it, only after a considerable delay, perhaps after from 6 to 12 days, or even after a longer period in the case of some species.

"2. Colonies on agar plates are very slow in appearing, perhaps after from 24 hours to 8 days, and the first colonies to arise are of minute size, having an average diameter of 0.2 mm. or less at the end of 96 hours on a favorable medium present in sufficient depth in the plate.

"3. The morphologic type of the organisms present in the filtrate, or in subcultures or colonies, is composed mainly of minute coccus forms, granular bodies and delicate skeins or filaments, with a suggestion of diplococcus or streptococcus arrangement (Giemsa staining); also occasional coccobacilli.

"4. In organisms the 'normal' form of which is acid-fast or gram-positive, the staining reaction of the recovered culture elements is temporarily reversed or variable.

"5. The biochemical and serologic reactions of the recovered cultures are different from those of the parent culture, and virulence, toxicity, and susceptibility to the homologous bacteriophage are altered."

Of course, contamination is ruled out by eventually regaining the original culture form after it has gone through its filterable stage.

### *Mucoid colony form (M)*

Moist colonies have been described for the diphtheria bacillus, but Hobby (1935) was the first to name a mucoid phase of the diphtheria bacillus. It has not yet been demonstrated, however, that diphtheria bacilli in the mucoid phase possess a capsule nor that they possess more of the type-specific substance than when in any other colony phase.

*Summary.* Thus, during the period of more than half a century that has elapsed since the original observations of the colonies produced by the diphtheria bacillus, many colony forms have been noted. These various colony forms were either described, or may now be interpreted, as the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D) and gonidial (G). Photographs and additional detailed descriptions of these different culture phases will be found in the paper by Morton (1940). The terms "mitis," "gravis," and "intermedius" as applied to the colony forms of the diphtheria bacillus do not truly designate the clinical manifestations which were originally thought to be associated with these cultural phases. Since the form of colony to which these terms originally referred can be described adequately by the older and accepted terminology (smooth and rough colony forms), there appears to be no need for the continuance of the terms "mitis," "gravis" and "intermedius" in that respect.

## 2. APPEARANCE OF THE GROWTH OF SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY FORMS IN LIQUID MEDIA

The manner of growth of a strain in broth often conveys as much information relative to the culture phase as does the appear-

ance of the colonies on solid media. In addition to confirming the observations of Roux and Yersin (1888) that diphtheria bacilli grow in broth as fine granules which adhere to the walls of the vessels, Brieger and Fraenkel (1890) observed that in some cases the bacilli grow to produce a homogeneous and uniform turbidity. The first indication of a correlation of such types of growth with differences in colony form in the diphtherial species came from the work of Baerthlein (1913) and of Bernhardt (1915) who reported that cells from a large, moist colony form grew with an even turbidity, whereas those from a fine, translucent, bluish, and brittle colony form grew only as a heavy, granular precipitate. The large, moist colony form described by Baerthlein produced a black color when grown on tellurite medium; and the fine, translucent, bluish form of colony produced only a light brown color. This represents the first indication that appearances on tellurite medium, in addition to the manner of growth in broth, may also be associated with colony form. Riemsdijk (1914) described three types of growth in broth, all of which can now be correlated with colony form: (1) granular growth on the bottom of the tube, associated with the rough colony form; (2) homogeneous clouding, associated with the smooth colony form, and type *mitis* colony described by the English workers; and (3) occasional pellicle formation, associated with the intermediate (SR) colony form and type *gravis* colony. The mucoid form is described by Hobby as growing with a uniform turbidity, similar to that produced by the S form. Neither the M, S, nor R forms grow in a granular pellicle (Hobby). These isolated observations have been confirmed by Morton (1940), who found in addition that the D form grows with a very faint turbidity, which is often finely granular. The G form often fails to impart visible signs of growth in filtrates for a considerable time. When visible signs of growth do occur, it is as a very fine turbidity with a ropy sediment. The statement in Bergey's manual that the diphtheria bacillus grows in broth as a "fine, granular deposit on sides and bottom of tube, forming a thin, fragile pellicle on neutral medium" is inadequate.

### 3. STABILITY OF THE CELL SUSPENSIONS IN SALT SOLUTION

The spontaneous clumping of diphtheria bacilli in physiological salt solution has often been very disconcerting. Indeed, Priestley (1911) reported that an agglutination test for the identification of *C. diphtheriae* was impractical since 12 out of 15 strains tested by him clumped spontaneously. The addition of glycerol, formalin, various salts, etc., failed to prevent the spontaneous clumping. Early, unsuccessful attempts were made to associate the property of remaining in uniform suspension in physiological salt solution with virulence or toxigenicity. Scott (1923) reported that such spontaneous clumping is not consistently associated with either the presence or absence of toxigenicity; therefore, it is not surprising that Okell (1930) and Jones (1930) were unable to find any significant relationship between agglutinability in salt solution and virulence. Although Scott found no complete association between roughness of growth and sensitiveness or insensitiveness to electrolytes, more recent studies have shown that sensitiveness to electrolytes is associated with colony form. Yü (1930) and Morton (1940) both found that the smooth colony form yields a uniform suspension in physiological salt solution. (The "mitis" form, which may be interpreted as a smooth colony form, gives uniform suspensions). The rough colony form was found to give granular and unstable suspensions. In a comparative study of this property among the S, SR, R, and D colony forms of diphtheria bacilli (Morton, 1940), it was found that the S and D forms give uniform suspensions in salt solutions in a concentration of 0.2 to 8 per cent, organisms in the R form clump spontaneously, and those intermediate between the S and R give indeterminate results.

### 4. VIRULENCE

It seems to be agreed that diphtheria bacilli, as identified by colony form, fermentation reactions, agglutination tests, etc., may be either virulent or non-virulent for guinea pigs, *i.e.*, the microorganisms, when injected into guinea pigs, may or may not bring about the death of the animals. The strains, whether



virulent or non-virulent, undergo the same variations in colony form. Earlier workers believed that diphtheria bacilli vary in virulence, ranging from non-virulent to highly virulent. Recent workers employing single-cell cultures and the intradermal method for estimating virulence find that diphtherial cultures are either virulent or non-virulent, none being intermediate (Powell, 1923; Dudley, 1925 and Okell and Parish, 1926). The work of Powell, in addition to showing that the virulence of diphtheria bacilli for guinea pigs varies only within narrow limits, showed that 40,000 to 400,000 organisms are required for a minimal reacting dose by the intradermal method, whereas 14 million to 140 million, or 35 times as many organisms are required when employing the subcutaneous method for estimating virulence. This, while establishing the superiority of the intradermal over the subcutaneous method, still leaves a rather wide latitude in the effective number of microorganisms.

Powell found that single-cell strains have the same degree of virulence as their respective parent cultures and different single-cell strains derived from the same single-cell strain are equally virulent for guinea pigs. However, since Roux and Yersin (1890) first reported that virulent diphtheria bacilli may give off non-virulent variants, other observers have noted the same phenomenon. In view of the fact that other species of virulent microorganisms give off non-virulent variants, the same process may be readily accepted for the diphtheria bacillus. Reports of the reverse process, that of non-virulent variants reverting to virulent forms, are far less common, and some believe that it cannot take place. Trumpp (1896) claimed to have transformed an avirulent strain into one possessing virulence by injecting the culture into guinea pigs along with sublethal doses of toxin. Morton (1940) reported that after two cultures of the virulent Park No. 8 strain had aged in ampoules 132 and 140 days, respectively, the organisms produced S colonies on solid medium whereas the strain originally was of the SR colony type. Upon five occasions, cultures from the S colonies were non-virulent for guinea pigs. After 14 generations on plain or blood infusion agar, one of the S strains killed guinea pigs in doses of 37,000,000 or-

ganisms administered intraperitoneally. An explanation for this reversion might be that during the physiological youth of the culture, it had not attained its maximum toxin-producing properties, just as was shown (Morton, 1940) that during the physiological youth of a diphtherial culture great variations in morphology may take place.

A study of colony forms has not contributed anything toward an explanation for virulent and non-virulent forms of the diphtheria bacillus. Cobbett and Phillips (1897) noticed no difference in virulence between the strains derived from their various colony forms. The dissociative studies by Hobby were made, unfortunately, on a strain which did not possess virulence. In the studies by Morton on virulent strains, the various colony forms (S, SR, R, and D) retained their pathogenicity for guinea pigs. Yü (1931) reported that his S, virulent form, isolated during the acute stages of diphtheria, was followed during convalescence by non-virulent S and R forms. Nevertheless, virulence is not associated with colony form as Yü thought it to be. This is evident from the studies by Cobbett and Phillips and by Morton, and is borne out by the reports cited in the next section on toxigenicity, where there are listed the results obtained from the use of culture filtrates instead of whole cultures.

It is rather instructive to examine the most recent description of the pathological effects of diphtheria bacilli in man as observed by McLeod, Orr, and Woodcock (1939) on the morbid anatomy in *gravis*, *intermedius*, and *mitis* diphtherias from a series of 51 necropsies. Their study indicates that an infection with type *mitis* (S form) diphtheria bacilli is of less serious menace to the individual than an infection with organisms of type *gravis* (SR form). In acute cases the primary lesions caused by types *gravis* and *intermedius* are differentiated from those of type *mitis* (S form) on the following points: much less superficial membrane in the fauces and less tendency to extend into the intrathoracic air passages; the membrane less firm in texture; deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues; and selective involvement of the tonsils and the deeper tissues at this site.

The most frequent causes of death were (a) respiratory obstruction as the result of membrane formation, which was more frequent with type *mitis*; and (b) toxic effects on the viscera, especially the heart and kidneys, which were more frequent with types *gravis* and *intermedius*. Thus, although virulence is not associated with colony form, the above evidence suggests that there may be a correlation between the colony form and certain pathologic characteristics.

Since the type *gravis* usually ferments glycogen in the test tube and type *mitis* does not, Knox and Passmore (1938) reasoned that the fermentation of glycogen might be the explanation for the greater virulence of type *gravis* within the human body. However, their finding by metabolic studies that both forms utilize glycogen eliminated this possible explanation. Povitzky, Eisner, and Jackson (1933) produced death in guinea pigs, weighing about 300 gm., in 24 to 48 hours with a Park No. 8, a type *mitis* and a type *gravis* cultures, employing the growth from  $1\frac{1}{3}$ ,  $\frac{1}{16}$ , and  $\frac{1}{3}$  veal-agar slants, respectively. Their explanation for the fact that the type *gravis* culture was more virulent than the others was that it probably produces toxin more readily in the animal body. Their *in vitro* results, however, do not bear out this reasoning, for in 48 to 72 hours, cultures from the above three strains contained 550, 350 and 200 M.L.D. of toxin per ml., respectively. Their *in vivo* results, in view of the findings of McLeod, Orr, and Woodcock, may be explained from another angle.

The vital question of why certain strains of diphtheria bacilli are virulent for man, guinea pig, or other animals, while other strains are not, is still unanswered. The property of virulence is not associated with colony form nor is toxigenicity the complete explanation. Perhaps careful chemical or metabolic studies will bring forth the long awaited answer.

## 5. TOXIGENICITY

In contrast to the previous section, wherein results of infection of the animal body with the various colony forms of the diphtheria bacillus were discussed, this section is concerned primarily with

the ability of these organisms to elaborate a toxin, as judged by testing the cell-free culture filtrate. Many of the technical details concerned with the production of diphtherial toxin have been described by Andrewes, *et al.* (1923). Investigators agree that diphtheria bacilli, as identified by colony forms, fermentation reactions, agglutination tests, etc., may or may not elaborate the specific exotoxin. Crowell (1926), working with a single-cell strain, observed that it is possible for a toxigenic strain to give off both toxigenic and non-toxigenic descendants. He, like others, never observed a non-toxigenic strain to regain its toxigenicity.

Toxigenicity is not necessarily associated quantitatively with virulence. It is generally known that the Park No. 8 strain, which produces one of the most potent diphtherial toxins, is not one of the most virulent strains. The work of Povitzky, Eisner, and Jackson (1933) showed quantitatively that the virulence and toxigenicity of different strains may vary independently of one another.

Toxigenicity is not associated with any serological type. Havens' (1920) report that the toxins elaborated by two serological types were not identical, created considerable consternation, for bacteriologists and clinicians had taken it for granted that there is but one diphtherial toxin. This report by Havens, however, was disproved by Paxson and Redowitz (1922), Hartley (1923), and Park, Williams, and Mann (1922). The latter workers found that the toxins elaborated by organisms of the various serological types are qualitatively, and from the practical standpoint, quantitatively alike.

It has been demonstrated that toxigenicity is not associated with colony form. The smooth and matt colony forms of Schick and Ersettig (1903) produced toxins which were neutralized by diphtherial antitoxin. Although Cowan (1927) and Yü (1931) claimed that their R strains failed to produce toxin, Povitzky, Eisner, and Jackson (1933) found that both types *mitis* and *gravis* produce toxin; and more recently Morton (1940) reported that the S, SR, R, and D colony forms produce toxin.

No matter in what serological group or colony form a diph-

theria bacillus may be, the toxin, if it is produced, is one and the same. There are, however, differences in the rate of production and the amount produced. The reason why certain strains of diphtheria bacilli are able to elaborate the exotoxin and other strains are not, is still unanswered. Dissociative studies and investigations into the antigenic structure of the bacterial cell have thus far failed to reveal the answer. Perhaps a study of the enzyme systems of the diphtherial cell will furnish an explanation.

## 6. SEROLOGICAL REACTIONS

Early investigators soon learned that there is no relationship between the agglutinability and the virulence or toxigenicity of diphtheria bacilli. Moreover, the agglutination reaction has failed thus far to reveal any changes in cellular antigens of virulent diphtheria bacilli when the bacilli become non-virulent. Also, it has not been possible to discover a diphtherial agglutinating serum capable of agglutinating a pseudo-diphtheria bacillus or a diphtheroid. Nor does a diphtherial agglutinating serum agglutinate all strains of diphtheria bacilli. The more this species is subjected to serological study, the greater is the number of serological types that are discovered. Lipstein in 1903 recognized four definite serological types of *C. diphtheriae*; and Durand (1918, 1920) reported five and a group of heterologous strains. Havens (1920) temporarily complicated matters by reporting only two serological groups but these findings were soon disproved by Bell (1922) and by Park, Williams, and Mann (1922). The latter found at least five serological types. Smith (1923) reported seven serological types; and Robinson and Peeney (1936), in a study of 739 strains, encountered a fifth type in addition to Ewing's (1933) four. Eagleton and Baxter (1923) found ten types, as did Sia and Huang (1939). Murray (1935c) reported eleven types and a group of unclassifiable strains. Undoubtedly more will be reported in the future.

Practically the same results have been obtained by agglutinin-absorption as by the agglutination test. The agglutinin-absorption procedure enables more strains to be classified because

spontaneous agglutination of the strains does not interfere with the text.

Only recently has the question of serological types been approached from a different angle of investigation, namely, by the attempt to isolate the chemical substance responsible for type-specificity. Wong and T'ung (1938) reported at first that the polysaccharides of *C. diphtheriae* are shared by types *mitis*, *gravis*, *intermedius*, and by an avirulent strain, and later (1939) that the polysaccharides are group-specific. It is quite possible that Wong and T'ung selected cultural variants of the same serological type for these studies. After Sia and Huang (1939) found different cultural forms within the same serological type, Wong and T'ung in their later work (1939, 1940) selected strains from different serological types, instead of from different cultural forms, for their chemical studies. As a result, they were able to isolate chemically a substance which reacts only with serum prepared against the homologous strain. To date the preliminary findings of Wong and T'ung are that the cellular constituent of the diphtherial cell which is responsible for serological type-specificity is an alkali-soluble protein that is heat-labile, and convertible to a group-specific protein by heating at 56°C. for 30 minutes.

In 1903 Schick and Ersettig reported that organisms from their smooth and matt colonies are agglutinated by the same anti-serum; and additional studies along this line by Morton (1940) showed that organisms in the S, SR, R, and D colony forms react similarly against type-specific serum. Single-cell strains from different colony cultures have the same agglutinative reaction as the parent strain (Powell, 1923).

The attempt on the part of the English workers to identify their types *mitis* and *gravis* by fermentation reactions and antigenic types appears to be impractical. It has been shown that microorganisms of one colony form may have the fermentation reactions of those of the other colony form. This, moreover, has been verified by studies in metabolism which reveal that microorganisms of both colony forms possess similar enzyme systems. Furthermore, it is not in keeping with our general

knowledge of bacterial species for different serological types within the species to be characterized by distinctive colony forms. In their investigations on the chemical substances within the diphtherial cell, Wong and T'ung found that a type-specific substance is present in strains of different cultural reactions, but it is present only in strains of the same serological type.

Inasmuch as the organisms in at least one of the major colony forms of the diphtherial species clump spontaneously in physiological salt solution and a fair percentage of diphtherial cultures encountered in nature give unstable suspensions, agglutinin-absorption or chemical fractionation of the organisms and precipitin tests, of necessity, have to be employed for serological typing. The chemical studies begun only recently by the Chinese workers should prove to be of great value. The early report that the bacilli from smooth and matt colonies are agglutinated by the same serum, and the preliminary finding that bacilli in the S, SR, R, or D colony forms behave similarly towards type-specific sera indicates that type specificity is not associated with a particular colony form, as is the case with *Diplococcus pneumoniae*.

## 7. HEMOLYSIS

It has been known since the report of Eijkman (1901) that diphtherial cultures are able to hemolyze red blood corpuscles, but that the hemolytic power is variable. The hemolytic substance is present in living cultures only, is destroyed by heat, and is non-filterable (Schwoner, 1904; Wesselow, 1914; Costa, Troisier, and Dauvergne, 1918). Practically all investigators have found that the hemolytic property in corynebacteria is associated only with the diphtherial species; strains of *C. xerosis* and pseudo-diphtheria bacilli lack this property. Red blood cells from different species of animals vary in susceptibility in the following decreasing order: rabbit, guinea pig, goat, dog, horse, and human (Schwoner, 1904; Goldie, 1933).

There is no correlation between the hemolytic power and the toxigenicity of a strain (Maunu, 1914; Heeren and Megrail, 1930), nor virulence nor the agglutination reaction (Hammer-

schmidt, 1924). Anderson, Happold, McLeod, and Thomson (1931) reported their type *mitis*, which we now know to be the smooth colony form, as hemolytic and their type *gravis*, which may be interpreted as an SR colony form, as non-hemolytic. The work of Christison (1933) brought out more strikingly the correlation of hemolytic power with colony form. She found that the rough strains derived from type *mitis* (smooth) colonies either fail to hemolyze red blood cells or give only faint traces of hemolysis. Smooth strains derived from type *gravis* colonies lyse red blood cells more frequently than do the rough strains.

In a comparative study of the hemolytic activity of the various colony forms, Morton (1940) reported that organisms in the S colony form are hemolytic, less so in the R, and the dwarf forms are non-hemolytic.

#### 8. FERMENTATION REACTIONS

Andrewes, *et al.* (1923) in their monograph on diphtheria give a historical survey of the development of fermentation tests for diphtheria bacilli and the diphtheroids. All workers agree that *C. diphtheriae* ferments glucose with the production of acid only, and that dextrin is usually fermented. Sucrose is usually not attacked; but Martin (1898), Cary (1917), Durand (1921), Fitzgerald and Doyle (1923), and Wright and Rankin (1932) have reported instances in which diphtherial strains have fermented sucrose. However, in view of the rarity of sucrose-fermenting strains of diphtheria bacilli, the readiness with which sucrose solutions may become altered during sterilization by heat or simply through the aging of aqueous solutions, and the diversity of media and pH indicators employed in fermentation tests, it might be well for this property to be reinvestigated by careful metabolic studies.

Schick and Ersettig (1903) reported that organisms from both their smooth and matt colonies fermented glucose; and more recently Cowan (1927) and Yü (1930) reported that their smooth and rough colony forms had the same fermentation reactions, the only difference being that the R form required a longer time to bring about the reaction. Judging from the descriptions or



photographs submitted by Cowan and Yü, it is not certain that they were working with the true S and R forms. At any rate, organisms in the S, SR, R, and D colony forms have the same fermentation reactions (Morton, 1940). Organisms from SR colonies are the most rapid fermenters, whereas the D forms require the longest period of time to bring about the production of acid. The differences between the various colony forms are quantitative rather than qualitative, a condition which is usually the case with the various colony forms within a species.

Anderson, *et al.*, (1931) observed qualitative differences in the fermentation reactions for different colony forms of the diphtherial organism. They reported inconsistent results with type *mitis*, while type *gravis* invariably fermented dextrin. Sharper differences, however, were encountered with starch and glycogen, which were fermented only by type *gravis*. The more vigorous fermentative activity of organisms in the SR colony forms (Morton) may explain the greater frequency with which type *gravis* attacked dextrin. Not always does type *mitis* (S form) fail to attack starch and glycogen, for Carter (1933) reported 1.17 per cent of the cultures from 510 positive swabs as being aberrant in that starch and glycogen were fermented but that the organisms grew in broth with a turbidity and no pellicle, a condition which is very suggestive of the S form. Robinson and Peeney (1936) also observed strains which were smooth and fermented starch, and strains which in other cultural respects resembled type *gravis* but failed to ferment starch. Ewing (1933) found two of 106 strains that fermented starch and produced type *mitis* colonies. Moreover, Wright and Rankin (1932) reported that none of their strains isolated from 50 cases of diphtheria fermented starch. They also found that one type *mitis* and two intermediate strains in addition to the type *gravis* strains fermented glycogen.

From the foregoing, one must conclude that the different colony forms of the diphtherial organism can not be differentiated definitely by qualitative differences in the usual fermentation tests. The clinching evidence for this decision would be metabolic studies with suspensions of the bacterial cells, and so far as the author is aware the only metabolic studies planned to clarify

this point have been those of Passmore (1938) and Knox and Passmore (1938). The latter workers found that there is no significant difference between the oxygen consumptions per hour per mg. dry weight of bacterial suspensions of types *mitis* and *gravis* when employing glucose or glycogen as the substrate. On the other hand, when tested in the usual way with glycogen in fermentation tubes, type *gravis* produces a strongly acid reaction, while type *mitis* remains neutral and, in addition, shows no utilization of the glycogen. According to Knox and Passmore, the discrepancies in results obtained when using fermentation tubes are accounted for by the differences in aeration within the tubes.

The property of utilization of carbohydrates by the diphtheria bacillus needs to be reinvestigated by more accurate metabolic studies. The great variations in manner of growth in liquid medium unquestionably account for great differences in aeration within fermentation tubes. The studies by Passmore show that enzymes for breaking down carbohydrates, when present in only small amounts, may be completely suppressed in organisms during their growth on nutritive media. At present there is no evidence substantiating the claim of Anderson, *et al.*, that the colony forms can be differentiated by qualitatively different fermentation reactions. The diphtherial organisms behave like other bacteria in that the various colony forms have the same fermentation reactions, though there are, perhaps, quantitative differences.

#### 9. CHROMOGENESIS

Numerous workers have observed the production of a pigment in diphtherial cultures. Andrewes *et al.* (1923) state that this property is probably more dependent upon the composition of the medium than upon the organism, but at the present time it is not known what is responsible for the pigmentation which is observed occasionally. A yellowish color was observed by Zupnik (1897), by Cobbett and Phillips (1897), and by Morton (1940). Baerthlein (1913) and Hadley (1927) observed the yellowish color among R variants while Baerthlein (1918) and

Ewing (1933) observed it among S strains. It is thus evident that pigmentation is not associated with any particular colony type, the pigmentation being observed in the S and R forms with about equal frequency. A citron-yellow color was reported by Przewoski (1912) and by Bernhardt and Paneth (1913) to be quite common. Heinemann (1917) found diphtherial cultures taking on a yellowish-brown color, as did Bernhardt (1915) and Hill (1903); the latter also reported a pink color.

No investigations were made upon the pigments until Smith (1930) observed in filtrates of diphtherial cultures a pigment which he identified as a porphyrin. He was not able to establish any relationship between the rate of formation of the pigment and the rate of toxin formation. Although Coulter and Stone (1931) reported a direct relationship between the biological titer of culture filtrates and porphyrin content, the work of Wadsworth, Crowe, and Smith (1935) suggests that the substances are not one and the same, because they observed that the "selective absorbing ingredient" could be removed from culture filtrates with activated charcoal without significantly lowering the toxicity of the filtrate. Also the results with an ultrafiltered toxin showed that the ingredient, which is soluble in ether, was present in both the toxic residue and the non-toxic filtrate. This pigment showed absorption bands similar to those of some of the porphyrins and appeared to be produced or liberated by the diphtheria bacillus under conditions also favorable for toxin production. These studies were carried out with toxigenic and non-toxigenic strains; the various colony phases of the organism were not touched upon. In a somewhat broader study, Wheeler (1940) found porphyrins present in cultures of 10 non-virulent, non-toxigenic strains of *C. diphtheriae*, of 3 strains of *C. ulcerans*, 2 strains of *C. ovis* and 1 strain of *C. hoagii* but not in cultures of 3 strains of *C. xerosis* and 1 strain of *C. hofmanni*. These observations are in accord with those of Wadsworth, Crowe, and Smith, in that the conditions under which pigment and toxin production are observed in cultures of virulent, toxigenic diphtheria bacilli are similar to those under which non-virulent bacilli and related species of *Corynebacterium* synthesize porphyrins.

## 10. REDUCTION OF POTASSIUM TELLURITE

The cells of *C. diphtheriae* become colored by the reduced metal when the culture is grown in the presence of salts of tellurous acid (Klett, 1900). Conradi and Troch (1912) proposed a medium containing tellurite for the isolation of diphtheria bacilli, because on this medium the colonies assumed a characteristic black color. Since then numerous authors have proposed methods and media based on this phenomenon for the rapid isolation of diphtheria bacilli from mixed cultures. Baerthlein's (1913) observation that a large, moist colony type produced a black color when grown on Conradi's medium, which Conradi considered typical for *C. diphtheriae*, and a fine, translucent, bluish type of colony produced only a light brown color is the first suggestion of the variation in the color of diphtherial colonies on tellurite media being associated with colony form. It is upon the appearance of the diphtherial colonies on potassium tellurite-chocolate agar that Anderson and his co-workers based their classification and judged the pathogenicity of the microorganisms for human beings. We have already seen that virulence and toxigenicity of the cultures are not associated with colony form. On the other hand, pigmentation of the colonies on the special tellurite medium is a property which can apparently be correlated with the colony form.

In connection with the reduction of potassium tellurite by diphtheria bacilli, Manzullo in 1938 described a rapid diagnostic *in vivo* test for diphtheria. His method is to touch the exudate or pseudomembrane in the back of the throat with a swab which has been dipped in a 2 per cent solution of potassium tellurite. In the case of diphtheria, the area thus treated is supposed to turn black in 5 to 10 minutes. In the original series of 75 patients, Manzullo obtained no false positive results and only 7.4 per cent of the cases of diphtheria gave negative reactions to the immediate tellurite test. The experiences of other investigators with this test have differed from that of Manzullo very strikingly. Fox, Rhoads, and Lock (1939) applied the test to 27 patients with throat infections. All of them gave a negative tellurite test in spite of the fact that *C. diphtheriae* was isolated on Loeffler's

medium from 17 of the patients. There was one false positive reaction among 10 cases of non-diphtheritic infections. Tomlin (1939) found 22.7 per cent false positives. Tombleson and Campbell (1939), in a study of 200 patients, reported agreement between the tellurite test and the bacteriological diagnosis in 67.5 per cent, and with the clinical diagnosis in 77 per cent of the cases. Definite blackening occurred in specimens from 84.3 per cent of the cases which were finally diagnosed as diphtheria. Blackening also occurred in 46.8 per cent of faucial lesions caused by organisms other than the diphtheria bacillus. In a study of 277 swabs from 84 patients, Cooper, Peters, and Wiseman (1939) experienced 22.8 per cent false negative reactions in 57 bacteriologically proven cases of diphtheria, and 55 per cent false positives in 27 patients in which *C. diphtheriae* was never demonstrated. Four strains of *C. diphtheriae* were isolated from Loeffler's medium which failed to appear on the potassium tellurite plates. Tynan (1939) reported 13 per cent false negative and 47 per cent false positive reactions in his study of 75 unselected patients. Murray (1939) in a study of 62 individuals found that 36.6 per cent of the non-diphtheritic infections in addition to 84.3 per cent of the diphtheria patients gave a positive Manzullo test. Of those individuals giving a negative test 20 per cent were definite cases of diphtheria. Among the series of 200 cases studied by Woodcock (1939), 113 diphtheria patients gave a positive swab in 91 per cent, and a positive Manzullo test in only 85 per cent of the cases. Of 39 non-diphtheria patients, 95 per cent gave a negative swab whereas only 21 per cent gave a negative test; of 48 doubtful diphtheritic infections, 73 per cent gave positive swabs and only 48 per cent gave a positive Manzullo test.

The consensus among those who have investigated the Manzullo test is that it is unreliable and cannot replace the bacteriological methods in use at the present time. It is not unreasonable to expect that, if colonies composed of diphtheria bacilli show a variation in the reduction of potassium tellurite, the pseudomembranes in the throat, also composed of diphtheria bacilli, will show a variation in the reduction of the tellurite salt. In

addition, one cannot ignore the fact that blackening of tellurite is a reduction process that may under suitable conditions be carried out by agents other than the diphtheria bacillus.

Anderson, Happold, McLeod, and Thomson reported that organisms from type *mitis* colonies were partially inhibited upon subculturing to chocolate tellurite agar. Morton (1935b) observed the inhibition of growth on chocolate tellurite agar of the S colony form as well as type *mitis*; and more recently Cooper, Peters, and Wiseman (1939), and Perry and Petran (1939) have cited instances where diphtherial organisms were isolated from Loeffler's medium but not detected on tellurite medium. If it is true that type *mitis* represents the smooth phase and type *gravis* some phase of the culture nearer the rough phase, then, on the basis of the properties associated with smooth and rough colony phases as cited by Hadley in 1927, one would expect type *gravis* to be more resistant to inhibitory substances than type *mitis*.

#### 11. CELL MORPHOLOGY

Many factors, both intrinsic and extrinsic, exert an influence upon the morphology of the bacterial cell. It was recognized early that there are many morphological forms of the true diphtheria bacillus; and Wesbrook, Wilson, and McDaniel (1900) attempted to classify the various forms, a classification which occasionally is used even today. They knew, of course, that cultures of *C. diphtheriae* are composed, not of one, but of many morphological forms. Only those forms which predominated, or were present in fairly large numbers were recorded for the culture. Almost all of the forms listed in their classification could be observed in any one culture if sufficient time was spent in the examination. Since the advent of the single-cell technic, the conclusion prevails that the various morphological forms of the diphtheria bacillus are of no hereditary significance, as the subcultures of a single cell of any morphological form may show a mixture of many morphological forms. This fact was demonstrated by Powell (1923) and Crowell (1926) and has been confirmed by all who have cultured single cells of this organism.

The various morphological forms that have been reported for the diphtherial organism may be listed as follows:

(A) *Rod forms.* By far the most common morphological form of the diphtherial organism is the rod. This form, however, is subject to a great many variations. Some of the factors which bring about such variations will be mentioned shortly. As a working classification, and based upon the appearances of the microorganisms after being stained with Loeffler's methylene blue, Westbrook, Wilson, and McDaniel grouped the morphological forms into granular rods, barred rods and solid-staining rods. Granular-barred forms were reported later (Schultz, 1909). These various rod forms may be grouped into four major groups, viz.: (a) granular rods, (b) granular-barred rods, (c) barred rods, and (d) solid-staining rods.

The significance of these forms is still a disputed problem, although the subject has received much attention because of its practical relation to the matter of diagnosis. These forms are readily changeable from one to another as numerous workers have shown. Wherry (1917) advanced the hypothesis that there is a normal evolution beginning with the  $D^2$  type or smaller and developing successively into the  $D^1$ ,  $C^1$ ,  $A^1$  and sometimes the A type (Westbrook classification). Others have agreed with this suggestion.

(B) *Thread and branching forms.* Long thread forms showing true branching were observed early in smears from diphtheritic membranes and in cultures. Egg media, serum agar, and potato media appear to favor the production of branching forms although they are observed in cultures on ordinary agar. Grasset and Grasset (1930) have described thread forms that were produced by growing the organism in the presence of bile. Thread forms from cultures that were suggestive of approaching roughness were seen by Neisser (1932) and similar forms, definitely associated with the rough colony forms, were described by Hobby (1935).

(C) *Spheroidal forms.* Under certain conditions it appears that the morphology of the diphtherial organism may assume a spheroidal form (Grubb and Koser, 1934). The coccus-like

cells of *C. diphtheriae* seem to be of two types: (1) those produced temporarily by the environment and not stable; and (2) those which seem to be stabilized and to be the result of some intrinsic change within the organism, such as the C-forms of Kuhn and Sternberg (1931) and the coccoid form reported by Stone and Hobby (1934).

The cell morphology of the diphtheria bacillus is of great practical significance because many tentative diagnoses are based upon it. One must be familiar with the influences which the colony phase and the environment may have upon the shape of the bacilli. Many of the factors are listed below.

*Relation to colony form.* When it was apparent that certain cultural characteristics often could be associated with colony form, it was to be expected that in the diphtherial species, as in many other bacterial species, certain morphological forms would show correlation with colony form. In the case of mucoid (M) colonies, no encapsulated organisms have been demonstrated. According to Hobby (1935), the organisms are longer and more granular than those obtained from smooth colonies. Organisms from smooth (S) colonies show more uniform rod forms than those from SR colonies. Although the cells are rod-shaped and stain unevenly, their outlines are not distorted to the same extent as are those from cells in the SR colony form (Morton, 1940). In the intermediate (SR) colonies, the cells are larger and the shapes more bizarre than is the case for cells from the other colony types (Cowan, 1927; Morton, 1935b). Organisms from the rough (R) colonies are long and filamentous, often forming long, intertwining threads (Hobby, 1935). Grasset and Grasset (1930) did not mention the colony phase in their report on the filamentous forms; but one recognizes that the cultures described by Neisser (1932) as filamentous forms ("Fadendiphtheriebazillen") were approaching the rough phase.

There is little information relative to the morphology of the organisms in the small colony forms prior to the studies described by Morton, (1935b), who found that organisms in the dwarf (D) colonies are short and somewhat thick with some tendency towards swollen forms and uneven staining by methylene blue.



Usually the cells stain solidly with the gram and methylene blue stains. They exhibit the typical arrangements of cells so common among corynebacteria. Organisms in the G colonies are very short, slender rods, sometimes nearly spherical. They are variable towards Gram's stain and stain unevenly with methylene blue.

Different sections of the same colony were observed by Zarniko (1889) to show variations in morphology and size of the diphtheria bacilli. This may be due to the differences in age of the various bacilli within a given colony or it may be due to the differences in the immediate surroundings of the bacilli in different sections of the same colony; both factors, as we shall see, are capable of exerting an influence upon cell morphology.

*Relation to age of culture.* Even during the normal growth of diphtherial organisms under the most favorable conditions, there is a great variation in the morphology of the cells. Many changes in morphology occur during the first 15 hours of growth on Loeffler's medium (Denny, 1903). The young cultures show solid-staining forms, and as the cultures grow older these forms give rise to larger and unevenly staining rods, which often show clubbing.

According to Clark and Ruehl (1919) who studied 70 strains of different species at brief intervals up to 48 hours and then at longer intervals for one week, striking changes in morphology take place during the early hours of growth. In all instances, except in the case of the diphtherial group, the organisms found in young cultures, 4 to 9 hours old, are larger than the forms found in 20- to 24-hour cultures. In the case of the diphtherial group, the young organisms, 2 to 6 hours old, are definitely smaller and more solid-staining than the older forms. Most of the strains do not attain the original size of the organisms inoculated until they are 12 to 18 hours old. These observations were, in part, confirmed by Henrici (1928) on a bacillus belonging to the diphtherial group. On the other hand, Albert (1921), working with 125 pure cultures of the diphtheria bacillus, did not corroborate the findings of Clark and Ruehl. He observed a slight increase in size during the first 4 hours, maintenance of that size

for about 20 hours, and then a rapid diminution after the 24-hour period. At the end of 7 days, the average length was only about one-third of the maximum. In regard to shape, the young bacilli, according to Albert, are rather bluntly pointed; with the development of granules, the ends become more rounded, even bulging. With the disappearance of the granules during the process of disintegration, the bacilli become irregular in shape with the ends again more pointed. Young bacilli stain solidly; but after a few hours, the protoplasm of some of the bacilli in certain areas takes a heavier stain than the intermediate portions, thus producing a barred appearance. The metachromatic granules appear in cultures 4 to 8 hours old. They are small at first, attaining their largest average size in cultures 12 to 15 hours old. After this period, the granules diminish in size and disappear. The percentage of bacteria containing granules increases rapidly from the 4-hour period, when there are but few granules, to the 12-hour period when 91 per cent of the bacteria contain granules. At the end of two days most of the bacilli have lost their granules.

Powell (1923) studied 299 single-cell cultures and found that they pass through different morphological phases similar to those derived directly from colonies. After a 4-hour incubation period on Loeffler's medium, the cultures contain only solid forms similar to the C<sup>2</sup> forms of Wesbrook. After 8 hours the morphology is still the same. After 12 hours the solid-staining forms still predominate but granular forms similar to the C forms begin to appear. After 24 hours all the cultures show a predominance of granular forms of the C type. A, B, and other granular forms are also present and some of the cultures show barred forms as well. During the next 24 hours the morphological picture remains about the same, except for the appearance of large irregular forms with many of the bacilli staining less distinctly with methylene blue. The single-cell cultures present the same morphological picture as do the corresponding colony cultures.

Customarily, observers view diphtherial cultures after 18 to 36 hours of incubation. In 1934, Solé introduced a method for

the rapid culturing of diphtheria bacilli; and since the reports of Brahdy *et al.* (1934, 1935) it is coming into greater vogue. For this method, sterile cotton swabs are impregnated with sterile, undiluted, unheated horse serum without preservative and heated over a flame to coagulate the surface. After swabbing the nose or throat, the swab is placed in a sterile, dry tube, incubated 2 to 4 hours and smears made directly from the swab. The diagnosis of such smears quite obviously necessitates a familiarity with the morphology of diphtheria bacilli in cultures 2 to 4 hours old, rather than the classical morphological pictures based on cultures 18 to 36 hours old.

*Relation to pH of the culture medium.* In acid media (+2 per cent), Denny (1903) observed that the organisms after 48 hours' incubation are short and sometimes oval in shape with well-marked granules. Alkaline media (-2 per cent) do not have as much effect on the morphology of the diphtheria bacilli as does an acid medium. Bunker (1917), on the other hand found that very acid media (pH not given) yield large, irregular, solid-staining forms in pure culture, while very alkaline media give minute, solid-staining, triangular forms, resembling Wesbrook's D<sup>2</sup> forms. Between these extremes practically all of Wesbrook's forms can be observed. Laybourn (1921) reported that acid media (about pH 6.1) give small, irregular forms with few granules, and that alkaline media induce minute, triangular forms with few or no granules. The observations of Morton (1935b) confirmed in part those of Denny and of Laybourn in that acid media (about pH 6.2) favor the production of small forms. However, a systematic examination of the effect of pH on the cell morphology of the diphtheria bacillus has yet to be made.

*Presence of glucose in the culture medium.* Heinemann (1917) and Yarisawa (1926) noticed that the presence of glucose favors the production of coccoid forms. This effect may perhaps be associated with the acidification of the medium as a result of the fermentation of the glucose; but acidification is not the entire explanation. Morton (1935b) confirmed these observations and in addition reported that media with a pH corresponding to that in tubes in which glucose is being fermented give small forms but these are not coccoid.

*Relation to oxygen tension.* Wherry (1917) observed that when a culture of the diphtheria bacillus is grown aerobically on Loeffler's medium the A<sup>1</sup>, C<sup>1</sup>, and D<sup>1</sup> types of Wesbrook predominate, whereas when the culture is grown anaerobically, the D<sup>2</sup> type predominates. In confirmation, Schneider (1931) and Beck (1933) likewise observed that anaerobic growth yields short, thick, solid-staining forms devoid of polar granules. In addition to studying the effect of oxygen on the morphology of *C. diphtheriae*, Beck studied the influence of other gases, as well. He found that H<sub>2</sub>, CO<sub>2</sub> and H<sub>2</sub>S, although without effect on the virulence of the microorganisms, do bring about changes in the morphology. The presence of hydrogen sulfide in the atmosphere results in a temporary increase in the rate of growth and in the formation of granules.

*Morphology in various media.* Coccoid forms of the diphtheria bacillus have been observed to arise in tryptic digest broth prepared from horse muscle (Parish, 1927); these forms reverted, however, to rod forms in the first subculture on Loeffler's medium. Parker (1928), employing tryptic serum agar, has noticed a somewhat similar phenomenon. Forty-seven per cent of 57 diphtherial strains are reported by Grubb and Koser (1934) as promptly changing their morphology to coccus forms when grown on liver infusion medium. Eighteen-hour cultures on inspissated horse serum are described as yielding short bacillary forms, whereas agar yields long forms (Barratt, 1924). The presence of blood in the medium is said to favor the production of granules (Megrail, 1922). Bile and bile salts are unique in that their presence in culture media is said to produce long filamentous forms with numerous branches and many metachromatic granules (Berthelot, Ramon, Grasset, and Amoureux, 1927; Grasset and Grasset, 1930). The presence of CuSO<sub>4</sub> in the culture medium has been observed to change the morphology of diphtheria bacilli to that of cocci (Pope and Pinfield, 1932; Morton 1935b, 1940). Morton has observed, in addition, that MgSO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, Cu(NO<sub>3</sub>)<sub>2</sub>, MgCl<sub>2</sub>, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> have somewhat the same effect.

The addition of guinea pig blood to agar favors the formation of coccoid forms (Yarisawa, 1926). Sterile emulsions of guinea

pig liver or kidney when added to agar have been noticed by Gins and Jermoljewa (1929) to change the morphology of diphtheria bacilli to that of the Hofmann bacillus. This change has also been observed by Schneider (1931) and Malcherek (1932). The first subcultures to Loeffler's medium from the peritoneal fluid of a guinea pig, that had been injected 24 hours previously by Crowell (1926) with a single-cell culture of diphtheria bacilli, have yielded a pure culture of coccoid forms. Subsequent transplants to Loeffler's medium from the culture of coccus forms have yielded pure cultures of rod forms. Maver (1931) has confirmed Hadley's (1907) observations that coccoid and solid-staining forms are produced when diphtheria bacilli are cultured in proteid-free media. Maver emphasizes that when there is a sudden change in the composition of the culture medium, the organism shows marked variations in morphology but that upon continued cultivation on the same medium the morphology gradually reverts to the more common rod forms. These observations may be the explanation for the change in morphology noticed when diphtherial microorganisms are cultured directly from the animal body. McGuigan and Frobisher (1936) recommend that the diagnosis of diphtheria should not be based upon the microscopical examination of smears made directly from colonies on tellurite medium, because the organisms often are small, thick, coccoid, and solid-staining. Involution forms have been reported as being not so abundant on amniotic fluid agar as on Loeffler's medium (Giltner and Ludlum, 1916).

*Temperature employed for sterilization of Loeffler's medium.* The amount of heat employed during the sterilization of the Loeffler's medium influences the morphology of diphtherial microorganisms when cultured upon it (Yarisawa, 1926; Morton, 1935b). The medium heated at 80°C. gives best coccoid formation, but when heated at 90°C. it yields typical rod forms. Perry and Petran (1939) also recognize the importance of the temperature of sterilization of Loeffler's medium in this connection.

*Morphology in a mixed culture with other organisms.* Smirnow (1908) in studying some symbiotic relations of *C. diphtheriae*

with other microorganisms has observed that such relationships give rise to coccoid forms. The change in morphology is not permanent as the diphtheria bacilli return to their typical morphology when grown under favorable conditions. Stovall, Scheid and Nichols (1923) have found that when a pure culture of *C. diphtheriae* is mixed and grown with a pure culture of *Staphylococcus aureus*, the diphtheria bacilli change their morphology from large club-shaped organisms with large granules to small thin organisms staining with heavy bands. No such effect is observed when non-hemolytic streptococci are substituted for the *Staphylococcus aureus* culture.

*Discussion.* From the relatively few citations of the voluminous literature it is evident that many factors, in the diphtherial organism itself, and in its environment, produce marked effects on the morphology of the cells. These factors have been cited because of their immediate practical significance.

In the study of the colony forms produced by a bacterial species, much information relative to the colony phase of the species often can be obtained by studying the morphology of the microorganisms. In the case of the diphtherial microorganism, however, many unusual circumstances are present. The genus *Corynebacterium* is exceptional in that during the normal growth and development of the microorganisms, the individuals are smaller during the first few hours of growth, then gradually attain their normal size. In the case of microorganisms of other genera the individual cells are slightly larger during the first few hours of growth. The so-called "normal" cells for the diphtherial species may be (1) rod forms, either granular, granular-barred, barred, or solid-staining; or (2) filamentous and branching forms; or (3) spheroidal forms. The significance of these various cell forms is not clearly understood at the present time. All conditions in the environment being as uniform as possible, the microorganisms in the R colony form are characterized by long, filamentous forms which stain solidly and often show branching. Microorganisms in the SR colony forms show a greater tendency towards bizarre forms; while those in the S colony form are more uniform in outline. The

organisms in the D colony form are characterized by their small size.

The diphtherial species is also outstanding in that the cell morphology is readily and markedly changed by the environment. The physiological youth and age of the culture, the reaction of the medium, the gaseous environment, the composition of the medium, and the temperature employed for sterilizing the medium have a decided influence upon the cell morphology. The sudden change of the microorganisms from one medium to another often results in a pronounced variation in the cell morphology. This variation is usually temporary, however, since continued subculturing upon one kind of medium results in restoring the microorganisms to their usual morphology. While many of the common factors that cause a temporary alteration in the cell morphology are known, there are doubtless many others still to be discovered.

#### SUMMARY

The diphtherial species, which in the modern conception of a bacterial species implies not only the diphtheria "bacillus" but all the cultural and morphological elements, is like many others in that it displays a variety of colony forms, namely, the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D), and gonidial (G) colony forms. The mucoid colony form, rarely reported, has not been demonstrated as yet to be made up of capsulated organisms or of organisms containing large amounts of a type-specific substance. The SR colony form, intermediate between S and R, occupies a greater rôle in the life history of the diphtherial species than similar forms usually occupy in other bacterial species. It is characterized by a pellicle type of growth in liquid media; such growth insures an adequate supply of oxygen to the growing organisms and thus enables them to develop more rapidly, and more readily to produce toxin or to ferment various carbohydrates. The R form is likewise a bit unusual in the diphtherial species in that the R colonies are smaller than the S, whereas in most other species it is the other way around. These various colony forms may be stable over a fairly long

period of cultivation or they may undergo spontaneous or forced variation. The trend in variation is from S to SR to R and reversion to the SR, or occasionally to the S form. Dwarf (D) colony forms have been observed to arise from the S, SR, and R forms. One is not able to predict with certainty what forms will arise when a given colony form is subjected to forced dissociation. In all cases, cultures from the S, SR, R, and D colony forms can be readily identified as the diphtherial species by their reactions towards the various test carbohydrates and specific immune serum, their virulence for guinea pigs, and their elaboration of the specific toxin.

Correlated with these various colonial manifestations is the manner of growth in liquid media. The M and S forms grow as uniform, homogeneous suspensions, the R form as a precipitate on the bottom of the vessel, and the SR form as a slight turbidity at first, which is replaced by a pellicle and sediment with the broth becoming clear. The D form grows as a very light turbidity and fine sediment, whereas the G form often fails to impart visible signs of growth in filtrates for a considerable period of time. When visible growth does occur, it is of a very fine turbidity with a ropy sediment. Suspended in physiological saline, microorganisms from the S colonies produce a uniform turbidity, those from the R colonies clump spontaneously, whereas organisms from the SR colonies give indeterminate results. Another property that appears to be correlated with colony form is the hemolytic activity of the cells. The S forms are hemolytic, the R forms less so, and the D forms non-hemolytic. Diphtherial cultures show the greatest hemolytic activity when 48 hours old; the hemolysin is thermolabile and non-filterable. Other members of the genus *Corynebacterium* have not been observed to possess this hemolytic property.

While the diphtherial microorganism reduces potassium tellurite, the various colony forms show differences in the rate of reduction and the degree of pigmentation. The differences in the amount of pigmentation of the colonies are probably due to the shape of the colony, the moisture content, and the texture of the surface. The S colonies (called "mitis" by the English



workers) which are raised, moist, and shiny appear black and shiny on tellurite medium. The SR colonies (called "gravis" by the English workers) are flatter and dryer, and appear grayish and dry on tellurite medium. The D forms which are slow in bringing about biochemical reactions are also slower than the S and SR forms in becoming pigmented.

Diphtherial microorganisms may be either virulent or avirulent for guinea pigs. If a strain is virulent, organisms from all the colony forms (S, SR, R, and D) may be virulent. There are, however, differences in the number of organisms required to produce death of the guinea pig in a stated time, the SR form having been found to produce death with fewer organisms than the S form. Quantitative tests have not been made with the R and D forms. In man, organisms from the S ("mitis") colony form appear to produce in the fauces a greater superficial membrane which is firm in texture and tends to extend into the intrathoracic air passages; and the most frequent cause of death is the respiratory obstruction produced by membrane formation. Organisms from the SR ("gravis") colony form appear to produce in the fauces less superficial membrane which is less firm in texture and shows less tendency to extend into the intrathoracic air passages; but this type produced deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues. In this case, the most frequent cause of death is the toxic effect on the viscera, especially the heart and kidneys. Toxicogenicity does not appear to be the complete explanation for virulence, since the most virulent strains are not necessarily the greatest or most rapid toxin producers. Diphtherial strains may be either toxigenic or non-toxigenic. If toxigenic, organisms in all the colony forms (S, SR, R and D) are capable of elaborating the toxin. There are, however, differences in the rate and amount of toxin produced. The important question of why certain diphtherial strains are virulent and toxigenic and other strains are not is still unanswered. These attributes of the diphtherial microorganism are not associated with any particular colony form, nor with a particular antigenic component or serological type, nor with any

known biochemical activity. Studies into the chemical composition and respiration of diphtheria bacilli have only recently begun, and should contribute valuable information on many of the obscure aspects of the physiology of the organism.

Another property of the diphtherial microorganism not associated with any particular colony form is serological specificity. Preliminary observations have shown that microorganisms in the S, SR, R, and D colony forms behave similarly towards type-specific sera. The antigen responsible for type specificity among diphtheria bacilli has not as yet been identified, but the chemical studies already under way by the Chinese investigators should furnish this much-needed information.

The fermentation reactions of the diphtherial microorganism do not vary qualitatively with the different colony forms, but there are variations in the rates and the amounts of acid produced. *C. diphtheriae* produces acid from glucose and usually not from sucrose, but there are exceptions. In view of the rarity with which sucrose is utilized and the uncertainties attending the older recorded instances of such utilization, a careful reinvestigation of this property by modern methods would be highly desirable. Acid is usually produced from dextrin, starch and glycogen, the latter two substances, especially, being more frequently attacked by organisms in the SR colony form than in the other colony forms, as judged by the usual fermentation tests. The first, and thus far the only, respiration studies of the different colony forms indicate that organisms in the S ("mitis") and SR ("gravis") colony forms alike utilize glycogen, whereas the usual fermentation tests show that only the SR forms utilize it, *i.e.*, produce acid. This sort of discrepancy emphasizes again the need for careful quantitative metabolic studies as a basis for reliable interpretation and characterization.

The cell morphology of the diphtherial microorganism is quite variable. In addition to the usual variations in morphological forms which accompany changes in colony form, extensive morphological changes take place during the normal growth of the microorganisms, changes that are greatly modified by the reaction of the medium, the composition and temperature of steriliza-

tion of the medium, oxygen tension and symbiotic relations with other microorganisms. In addition to showing a variety of sizes and shapes, the microorganisms may appear as granular, granular-barred, barred or solid staining rods which may show filaments or branching forms. They may also appear in spheroidal form which, in certain cases, is transitory and dependent upon the environment, and in others is more permanent and independent of the environment.

This review of the many isolated observations of earlier investigators and of the results of more recent studies on the diphtherial organism emphasizes the necessity for viewing the diphtherial species in a manner quite different from that heretofore demanded by the older monomorphic concept of a bacterial species. *C. diphtheriae* is not typified exclusively by the "bacillus" of Klebs and Loeffler but by a considerable diversity of morphological and cultural elements that, taken collectively and perhaps in a certain sequence, make up the diphtherial species.

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# NON-SPOREFORMING ANAEROBIC BACTERIA OF MEDICAL IMPORTANCE

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The non-sporulating anaerobic bacteria, although discovered at about the turn of the century, are as little known to most bacteriologists today as they were thirty years ago. The textbooks of bacteriology give little if any discussion of them, so that it becomes a major research problem for the laboratory worker to identify any of these species when encountered. The importance of the non-sporulating anaerobic bacteria in medical bacteriology is controversial. At the Third International Congress for Microbiology in the section on Anaerobes the following statement was made by Thompson (1): "In clinical bacteriology, anaerobes play a minor role. The finding of anaerobes is analogous to the occurrence of red-letter days on the calendar—when they occur they are usually worthy of consideration." With this statement in mind, inquiry was made regarding the incidence of non-sporulating anaerobes in specimens submitted for bacteriological examination in the Department of Surgery at the University of Chicago, Billings Hospital.<sup>1</sup> From July, 1936 to May, 1940, the following results were obtained:

SPECIMEN	TOTAL NUMBER OF SPECIMENS EXAMINED	NUMBER OF SPECIMENS CONTAINING NON-SPORULATING ANAEROBES	PER CENT CONTAINING NON-SPORULATING ANAEROBES
Pus.....	2,621	146	5.57
Fluids.....	786	18	2.29
Tissues.....	428	16	3.73
Gall bladder wall.....	340	13	3.82
Bile.....	371	3	0.80
Blood.....	634	4	0.60
Total.....	5,180	200	3.86

<sup>1</sup> The author is indebted to Miss Irma Holicky, Bacteriologist, Department of Surgery, University of Chicago, for collecting these data.

The 200 specimens contained 230 non-sporulating anaerobes as follows:

	GRAM- VARIABLE	GRAM-POSITIVE	GRAM- NEGATIVE
Staphylococci.....	0	4	0
Streptococci.....	1	$\alpha$ 13, $\beta$ 4, $\gamma$ 45	5
Black colonies (Strep.).....	0	1	0
Coccobacilli.....	0	4	6
Rods.....	6	9	46
Fusiforms.....	0	0	59
Diphtheroids.....	0	24	0
Curved diphtheroids.....	0	3	0

Of the 200 specimens containing non-sporulating anaerobes, 54 contained them in pure culture, 20 in mixture with other anaerobes, and the remaining 119 in mixture with both anaerobes and aerobes. It is obvious from the above figures that non-sporulating anaerobes were encountered in routine surgical bacteriology on the average of about 50 times a year and were present in approximately 4 per cent of the total specimens examined. This incidence is too high to be ignored in any routine cultural studies.

McCoy and McClung (2) in their bibliography of the anaerobes list some 266 species of non-sporulating anaerobes with a total of 831 references. Of the 266 species, however, only 16 have received much attention. Two of these may be omitted from this discussion, one, *Bacteroides bifidus*, is more properly grouped with the lactobacilli, and the other, *Spirillum desulfuricans*, is not of importance in medical bacteriology. The remaining 14 with which this review is concerned are as follows (the numbers in brackets following each organism represent the number of references listed for the species):

*Staphylococcus parvulus* [45]  
*Streptococcus anaerobius* [17]  
*Streptococcus foetidus* [25]  
*Streptococcus putridus* [30]  
*Bacteroides fragilis* [63]  
*Bacteroides funduliformis* [72]  
*Bacteroides furcosus* [15]

*Bacillus fusiformis* [151]  
*Bacterium melaninogenicum* [18]  
*Bacillus nebulosus* [15]  
*Bacterium necrophorum* [64]  
*Bacterium pneumosintes* [47]  
*Bacillus ramosus* [56]  
*Bacteroides serpens* [17]

Several generic names are cited for a single species, which is in keeping with the confusion now existing in bacteriological nomenclature. Recently Prévot (3) has proposed new generic names for the anaerobes. Bergey's Manual (3a) classifies the gram-negative members of this group under the generic name of *Bacteroides*. Since these organisms are so different in morphology and physiology, it seems absurd to group them under one genus. In this review mention will be made of some of the generic and specific names applied to these microorganisms, but no attempt will be made to name them. As will be pointed out subsequently, we do not know enough about many of them to assign them to special genera.

Most of the work on the non-sporeforming anaerobes has been done by clinicians, who have isolated and described new species. The descriptions of many species have been so inadequate that the same organism may be again discovered by another investigator and given a new name. Another difficulty is that no two workers will use the same technique in isolation and identification, thus affording no comparative basis for the examination of newly isolated strains. In many hospitals no effort is made to look for this group of organisms, hence the laboratory diagnosis of "sterile pus" is made on specimens from abscesses, if no growth appears on aerobic blood-agar plates.

Nearly all the non-sporulating anaerobes of medical importance are normal inhabitants of the mucous membranes of the body, inhabiting the upper respiratory tract, the colon and the genital tract. Under conditions which give rise to necrosis of the mucous membranes one or more of these species may become established and invade the tissue. It is when they produce abscesses or enter the blood stream that they are usually detected. They are not found at their original portal of entry because of poor methods for their isolation. Most workers in bacteriology seem to think that a search for anaerobes is too arduous a task to attempt, although with modern apparatus it is possible to isolate and study anaerobes in relatively convenient routine fashion. In the author's experience the early method proposed by Veillon for the isolation of anaerobes is entirely inadequate when the

anaerobe sought is greatly outnumbered by other bacteria. No originality is claimed for the method used successfully in this laboratory for many years. The method is as follows: A blood-agar plate containing 10 per cent of sheep blood is streaked with specimens to be examined. When the specimen is heavily contaminated a second plate is streaked from the first. Anaerobic conditions are provided by using ordinary pyrex desiccators with ground glass stoppers. The ground joints of the jar are sealed with a preparation made of equal parts of rubber, paraffin, and vaseline. This sealing preparation is effective at incubator temperatures. The plates after streaking are placed in the jar over a solution of pyrogallic acid and sodium carbonate. The jar is closed and evacuated with an oil pump to approximately 10 cm. mercury pressure. Carbon dioxide is allowed to flow into the jar until atmospheric pressure is reached, after which the jar is again exhausted to 25 cm. mercury pressure and is then sealed and placed in the incubator. Oxygen dissolved in the blood agar is absorbed by the sodium pyrogallate. The blood-agar assumes a cyanotic color when good anaerobic conditions are attained, thus serving as an indicator of the relative absence of oxygen in the jar. Non-sporulating anaerobes of diverse types have been successfully isolated by this simple procedure.

#### ANAEROBIC COCCI OF MEDICAL IMPORTANCE

Numerous species of anaerobic cocci have been described, but only four have been sufficiently studied to warrant space in this review.

*Staphylococcus parvulus*. This organism has also been listed under the genus *Micrococcus*, although Weinberg *et al.* (4) have placed it in a genus *Veillonella*, because it is gram-negative and differs from the members of the genus *Neisseria* in having an interstitial substance (ectoplasm) which may be demonstrated by Giemsa's stain. This organism was first described by Veillon and Zuber (5), who claimed to have found it in pus in appendicitis, either in the abscesses about the cecum or in the peritoneal cavity in a generalized peritonitis. Subcutaneous abscesses have been produced in rabbits and guinea pigs with these strains.

These workers noted the predominance of this organism over *Escherichia coli*. All cultures produce gas and a very foul odor. Gelatin is not liquefied and milk is not affected. Glucose is fermented and certain strains apparently attack other carbohydrates (3, 4), although there seems to be considerable irregularity in this respect.

*Streptococcus anaerobius* and *Streptococcus foetidus*. These two species are listed together, since the description of their activity in different media is identical. The only difference ascribed to them is in morphology, and with the recognition of variation among other species of bacteria, it does not seem at the present time that this difference is in itself adequate to separate these as two distinct species. *Streptococcus anaerobius* is described as having long regular chains, whereas *Streptococcus foetidus* appears in short chains and irregular arrangement with occasional tetrads. *S. anaerobius* was first described by Krönig and Menge (6) and *S. foetidus* by Veillon (7).

Both organisms are gram-positive and are generally found in the oral cavity, intestine and vagina in abundance. They produce gas and foul odors in all media. Gelatin is not liquefied and neither milk nor heat-coagulated proteins are affected, although good growth occurs in serum and a very foul odor results. In man, the organisms have been found in purulent gangrenous processes involving the genital tract, lungs and viscera, and in septicemias. They do not produce strong toxins, neither do they cause hemolysis. There seems to be a difference of opinion among workers regarding their pathogenicity for animals. Prévot (3) claims that local edematous lesions with gangrenous suppuration have been produced in guinea pigs with *S. foetidus*.

*Streptococcus putridus*. Schottmüller (8) first described *Streptococcus putridus* and reported 25 cases in which it was found in the lesions. These cases comprised meningitis, cystopyelitis, gangrene of the lung, salpingitis with pelvic abscesses, and septic abortion (ten cases, of which eight had thrombophlebitis with other complications). *S. putridus* was isolated in pure culture in 12 of the cases, and found along with other organisms in the rest.



Schottmüller observed that on artificial media long and short chains were present and the individual organisms were usually flattened and appeared attached as diplococci. In old cultures he noticed different shapes, with some cells appearing as rods. The strains were all gram-positive. *S. putridus* was often isolated from blood by placing the blood immediately in bouillon and incubating it without mixing or further handling. On blood-agar plates the colonies were porcelain white in color, and of the size of the head of a needle.

The main characteristic by which *S. putridus* may be differentiated from other anaerobic streptococci is the reaction in blood-broth cultures. The blood takes on a characteristic poppy-red color; and spectroscopically  $H_2S$  may be demonstrated in blood cultures. In about ten days blood-broth cultures are black in color. Schottmüller believed *S. putridus* was not a simple saprophyte, since it was able to invade and produce lesions elsewhere in the body. He called attention to the ability of this organism to dissolve fibrin, since in the pleural exudates one never finds the slightest trace of fibrin clots.

*S. putridus* is strictly anaerobic. Heat-coagulated protein media are not attacked, whereas sterile protein solutions such as serum are attacked and give rise to a foul odor. Glucose, levulose and maltose are fermented (3).

Since Schottmüller's original description of *S. putridus* in 1910 (8) there has been little attention given this organism. A few reports have appeared, some of which will be mentioned. Schwarz and Dieckmann (9, 10) in St. Louis made a search for this organism in puerperal infections. Of 165 uterine cultures and blood cultures from suspected cases, they found that 46 contained *S. putridus* (9). More recently Stone (11) has studied 26 strains of anaerobic streptococci, which he isolated from parturient and post-abortal women by means of a sterile pipette inserted through the cervix into the uterus. The organisms were found to be gram-variable. Stone attempted to apply some of the tests used in differentiating aerobic streptococci of the beta-hemolytic type, such as growth in 10 and 40 per cent bile, final pH in glucose medium, hydrolysis of sodium

hippurate, fermentation of trehalose and sorbitol, and finally the precipitin reactions. He found that it was impossible to set up definite groups of these strains by means of their precipitin reactions. Stone made no attempt to differentiate *S. putridus* from other anaerobic streptococci by its reaction in blood-broth, as recommended by Schottmüller (8).

#### ANAEROBIC NON-SPORULATING GRAM-NEGATIVE RODS

Organisms of this group have been placed in many genera. Castellani and Chalmers (12) have listed a Tribe *Bacteroidae* in the Family BACILLACEAE with the following description: "Bacillaceae with good growth on ordinary laboratory media, without endospores, fluorescence, or pigment formation, and obligatory anaerobes." They named a genus *Bacteroides* with these tribal characters and *Bacteroides fragilis* was given as the type species. This generic name, *Bacteroides*, includes both gram-negative and gram-positive species, although it is proposed (13) that it be restricted to the gram-negative species.

From the author's experience with the gram-negative non-sporulating organisms, it seems unwise to place all of these in a single genus, since they represent greatly different morphological types. In this review, however, these organisms will be listed under the names appearing in the McCoy and McClung bibliography (2).

#### *Bacteroides fragilis*

Prévot (3) has listed this organism in a genus which he calls *Ristella*, and defines as containing asporulating simple rods, non-ciliated, non-motile, straight or slightly curved, non-capsulated and gram-negative. Under this genus he has listed 25 species. Topley and Wilson (14) list this organism in a genus *Fusiformis*, which they characterize as "obligate parasites, anaerobic or microaerophilic. Cells frequently elongated and fusiform, staining somewhat unevenly. Filaments sometimes formed; non-branching. Non-motile. No spores. Reaction to Gram variable. Growth in laboratory media feeble." Veillon and Zuber (5) called this organism *Bacillus fragilis*. In addition

to having four different generic names, *Bacillus*, *Bacteroides*, *Fusiformis* and *Ristella*, it has another species name: *sassmannshausen*, given it by Heyde in 1911 (4).

The description given this organism by Veillon and Zuber (5), who found it in 22 cases of appendicitis, may be translated as follows:

"This bacillus appears to us to be the most abundant and the most constant in the pus from appendicitis. It is a fine rod, a little smaller than that of diphtheria, rounded at the ends and regular. It presents itself in the form of rods, isolated or united two by two by one of their extremities. Sometimes certain bacilli are slightly curved. In culture they have the same appearance, although they appear a little larger and certain rods are longer. It is gram-negative and non-motile. Although this bacillus is in great abundance in the pus from appendicitis, it is difficult to isolate. At incubator temperature the colonies do not appear in the depth of the agar until the third or fourth day. They form little round or slightly irregular colonies, ovoid, brownish yellow, rather opaque with smooth borders. These colonies remain discrete and although they are not far separate, one from the other, they remain punctiform. The most isolated colonies are less than one millimeter in diameter, and it is necessary to transplant them as soon as they are evident because they die quickly. A culture left 7 or 8 days in the incubator is no longer viable. On agar at the surface this organism forms extremely fine colonies, very transparent, grayish, scarcely more marked than those of pneumococcus and, like those, at the end of several days they become less visible and seem to be reabsorbed.

"Cultures can be obtained on gelatin at room temperature. The colonies which appear at the end of 8-10 days are punctiform, yellowish granules with wet edges. The medium is not liquefied. The culture is viable for 20-30 days. In broth growth occurs easily and in relative abundance. The medium is uniformly cloudy and there is a fine whitish deposit at the bottom of the receptacle. The cultures do not give off enough gas to break up the agar, but do give off a fetid odor. We have not established the production of spores and, as we have said, this bacillus is very fragile and non-resistant. It is pathogenic for guinea pigs and forms abscesses when injected subcutaneously. If the animal does not die of its abscess, the pus is eliminated and the guinea pig becomes cachectic and dies in about a month. This bacillus is much more virulent for rabbits. By subcutaneous inoculation there is produced a

large phlegmon with separation of the skin and death in 6 to 7 days. An inoculation in the veins produces death by cachexia; but one is unable to find the bacilli in the body. It is probable that toxins are the agents in this case, because one obtains the same results with dead cultures."

This description given by Veillon and Zuber is useful in the isolation and identification of the organism. Cohen (15) listed this organism in 4 out of 16 cases of abscess of the lung, although his summary stated that he found it five times. His strains fermented maltose, glucose, sucrose and lactose, but not mannitol and inulin. Litmus milk was not acidified and gelatin not liquefied. Henthorne, Thompson and Beaver (16) isolated strains from the following: pelvic abscess in a patient with carcinoma of rectum, hepatic abscess and appendix in a patient with gangrenous appendicitis, abscess over sacrum in a patient with pilonidal cyst, and from the appendix in gangrenous appendicitis. Three of these cases were fatal. Their strains fermented glucose, maltose, lactose, sucrose, levulose, inulin, dextrin, xylose, raffinose, galactose and glycogen. Only one of the 4 strains fermented rhamnose, arabinose and trehalose, whereas none of them fermented mannitol, inositol, dulcitol, glycerol, salicin or sorbitol. They did not produce  $H_2S$ , hemolysis on blood-agar plates, reduction of nitrates or liquefaction of gelatin. Acid coagulation was produced in milk and gas was produced in the carbohydrates that were fermented.

*Bacteroides fragilis* is not limited to lesions about the appendix or the intestinal tract. It has been found, as previously mentioned, in lung abscesses as well as in many other conditions, such as periurethral and other infections of the urinary tract. It has also been found in septicemias with metastatic abscesses. No toxins have been demonstrated, in spite of the suggestion of their presence by Veillon and Zuber.

Not all workers are in agreement with Veillon and Zuber regarding the pathogenicity of these strains for rabbits and guinea pigs. The problem of differences of opinion concerning pathogenicity will be discussed in the review under *Bacterium necrophorum*.

*Bacteroides funduliformis* and *Bacterium necrophorum*

These two organisms are grouped together, since they have common properties and there seems to be no good reason for classifying them separately. Damman (17) in 1884 probably saw *Bacterium necrophorum* in the lesions of calf diphtheria. Loeffler (18) in the same year observed the organisms in calf diphtheria and succeeded in producing necrotic lesions in mice by subcutaneous inoculation of the necrotic membrane. He obtained a primary isolation of the organism from mice on calf serum but failed to subculture it. In 1891 Schmorl (19) reported an epidemic among rabbits in his laboratory, which was characterized by caseous necrotic lesions of the mucosa. He isolated an organism from the lesions which he named *Streptothrix cuniculi*. Much work by veterinary bacteriologists followed these early studies and consequently infections in animals caused by *Bacterium necrophorum* are commonly recognized.

*Bacteroides funduliformis* may have been first studied by Veillon and Zuber in 1894 (5) and described by them as species C. The first clear-cut recognition of this organism was by Hallé (20), who found it in the vagina in the healthy state, in exudates from retained placentas, and in pus in Bartholinitis. He described the organism in pus as a rod, generally slightly curved. He observed that when it is the only organism in pus it is never very abundant, that it does not stain well and that sometimes its ends are better colored than its center.

*Bacterium necrophorum* has received numerous names, which follow (3, 4):

- Bacillus of Schmorl (Weinberg et al.)
- Bacillus necrophorus* (Flügge)
- Actinomyces necrophorus* (Bergey, 1930)
- Bacillus necrosus* (Jensen)
- Bacillus diphtheriae vitulorum* (Flügge)
- Bacillus filiformis* (Schütz)
- Nekrosebacillus* (Bang)
- Streptothrix cuniculi* (Schmorl)
- Actinomyces cuniculi* (Gasperini)
- Bacillus necroseos* (Salomonsen)
- Bacillus des Kälbernoma* (Ritter)
- Streptothrix necrophora* (Kitt)

*Actinomyces necrophorus* (Neukirch)  
*Corynebacterium necrophorum* (Lehmann and Neumann)  
*Fusiformis necrophorus* (Topley and Wilson)  
*Corynebacterium de la necrose* (Hornach)  
*Spherophorus necrophorus*

The names given *Bacteroides funduliformis* (4) are:

Espèce C (Veillon and Zuber, 1894)  
*Bacterium funduliforme*  
*Bacillus funduliformis*  
*Bacillus thetoides* (Rist and Guillemot, 1898)  
*Spherophorus funduliformis*

This multiplicity of names is sufficient evidence for the confusion in the isolation and identification of these organisms. Prévot (3) has recently given the family name SPHEROPHORACEAE to the gram-negative organisms in the Class ACTINOMYCETALES. He has given the generic name *Spherophorus* and defined it as follows: Rods, straight or slightly curved, very polymorphic, occurring in exudates, ovoid with bipolar staining, in cultures forms are variable: filamentous, swollen, in form of sausage, ramified with constant presence of spheroids of variable shape, sometimes very large, metachromatism in elongated and filamentous forms, non-motile, non-ciliated, non-sporeforming and gram-negative.

This generic description by Prévot is sufficient excuse for the many names which these organisms have received. Animal and human strains of *Bacterium necrophorum* grow well on the surface of anaerobic blood-agar plates, prepared according to the method previously described. When the plates are removed from the anaerobic environment and exposed to air, a greenish zone appears about the colonies, which upon prolonged exposure to the air may change to a clear hemolysis. In the anaerobic state when the hemoglobin is reduced no hemolysis may be seen about the colonies. Colonies vary in size on different anaerobic blood-agar plates; sometimes they appear very small and at other times large. No reason can be ascribed for this condition. There is no significant difference in the colonies from human and animal origin (21). A foul odor is produced in all cultures.

The morphology of the cells of *Bact. necrophorum* is variable, as Prévot (3) has stated in the definition of the genus in which he places these organisms. The morphology varies with the type of medium used, so that it may even be questioned whether cultures are pure (Hallé, 20). In general, animal strains produce long, filamentous forms in broth and in anaerobic blood-agar slant cultures, whereas human strains have more "ghost forms" and short forms. Irregular staining and granules are commonly found in the cells. The morphological difference, however, is not absolute or clear-cut enough to warrant making species differentiation. All strains are gram-negative.

*Biochemical reactions.* Many difficulties are encountered in determining the biochemical properties of this group of bacteria. Some strains fail to grow in a basic medium of veal infusion broth or they grow with great irregularity, unless a fermentable carbohydrate is present. The addition of 10 per cent serum, 0.05 per cent cystine or 0.1 per cent cysteine has been found effective (21) in supporting growth in the basic medium. Glucose, maltose and levulose are fermented and, in general, more acid is produced from glucose and levulose than from maltose. Lactose, sucrose, mannitol and glycerol are not fermented and litmus milk is unchanged. Indole is produced in tryptophane veal infusion broth containing 0.05 per cent cysteine. Gelatin is not liquefied and none of the strains digests coagulated egg white. Many strains cause a drop in pH of only about 0.1 in the basic medium of veal infusion broth containing 0.05 per cent cysteine and frequently produce a small amount of gas in solid agar medium. The irregularity of growth due to the sensitivity of these organisms to oxygen, together with the property which some of them possess of producing slight amounts of acids and gas in basic medium, probably accounts for the lack of uniformity in the reported biochemical reactions.

*Pathogenicity for animals.* When strains isolated from many animal lesions are injected subcutaneously into the rabbit, a spreading necrotic lesion develops which kills the animal in 6 days or longer. Not all strains are lethal in this way. Orcutt (22) found that one of 10 cultures isolated from a bovine liver

abscess produced only a local abscess upon subcutaneous injection into a rabbit. Some of the human strains produce spreading lesions and death, but usually only local abscesses (23, 24) which are slow in healing. Organisms may be found in the pus for long periods of time (23). Intravenous injection of human strains sometimes gives rise to joint lesions.

In experimental infections in rabbits sulfanilamide has given good therapeutic results (25), although in certain infections in man the results have been discouraging (26).

The guinea pig appears to be quite resistant to human strains of *Bacterium necrophorum* (27). This is in contrast to the results of Hallé (20), who reported abscesses from subcutaneous injections. Guinea pigs on a vitamin C deficient diet, however, readily develop lesions when injected with human strains (27). It may well be that the success which early investigators had in producing lesions in laboratory animals was due to the deficient diets of their experimental animals.

*Immunological reactions.* The problem as to whether *Bacterium necrophorum* produces toxins is one that has been much debated. Beveridge (28) in a study of 12 animal strains concluded that these organisms produce a soluble toxin and an endotoxin, the latter being resistant to heat and chemical agents. He claimed to demonstrate exotoxins by filtering a broth culture and injecting rabbits intradermally with 0.1 ml. of filtrate. Subcutaneous inoculations of rabbits with 1 to 3 ml. of filtrate produced no obvious local reaction, but a slight hemorrhagic appearance to underlying muscles was observed through the skin. Four milliliters of a Berkefeld N filtrate given intravenously to a 2000-gram rabbit killed the animal in one hour, whereas 3 ml. of a "Seitz EK special" filtrate similarly given to a 1500-gram rabbit caused collapse in one hour, but the rabbit survived. In guinea pigs, 1.5 ml. of a fresh Chamberland L3 filtrate inoculated intravenously or subcutaneously had no effect, whereas 0.1 ml. intradermally produced only very slight swellings 0.5 cm. in diameter. One to 2 ml. of whole culture intravenously killed 3 of 4 guinea pigs in from 4 to 20 hours. A sheep inoculated intravenously with 20 ml. of fresh Berkefeld N filtrate



developed diarrhea, labored breathing and anorexia for 3 days, then recovered.

Beveridge (28) demonstrated endotoxins by treating suspensions of the organisms in different ways, such as (a) formalinizing (0.5 per cent) and incubating for one and for 8 weeks at 37°C., (b) heating at 100°C. for one hour, and (c) heating at 60°C. for 15 minutes. All of these preparations, when inoculated intradermally into rabbits in doses of 0.1 ml., produced well-marked nodular swellings 0.5 to 1 cm. in diameter and necrosis of the deep layers of the skin. The swellings persisted for several weeks.

The soluble toxins which Beveridge demonstrated were certainly not very strong. Scrivner and Lee (29), on the other hand, prepared filtrates from pure cultures of *Bacterium necrophorum* strains of animal origin and found that they did not contain sufficient toxin to affect rabbits injected subcutaneously or intraperitoneally. Furthermore, they failed to find a toxin sufficiently strong to affect calves when the filtrates were injected subcutaneously. They found that immunization with a filtrate was of questionable value in protecting rabbits and calves from artificial infection with the organism.

Filtrates from human strains of *Bacterium necrophorum* when injected intravenously into rabbits may give rise to some loss in weight, but the toxicity of such filtrates is not very marked. No satisfactory immunity is built up against infections with this organism. Rabbits immunized with human strains develop abscesses when injected with living cultures just as readily as non-immunized animals. With strains of animal origin, vaccination has been unsuccessful (28).

The various strains of *Bacterium necrophorum* do not form a homogeneous agglutinating group, such as occurs in the case of *Eberthella typhosa*. Many of the strains have agglutinogens which are unrelated, so that a single agglutination test is insufficient for identification of these organisms (24, 28, 30, 31).

*Bacterium necrophorum* infections in man. Numerous reports have been made of infections of man with this organism. Schmorl (19) and one of his assistants each developed a small

abscess on one finger while working with their *Streptothrix cuniculi*. Harris (32) described an anaerobic organism, *Bacillus mortiferus*, that he isolated from a liver abscess in man. *B. mortiferus* has many of the features of *Bacterium necrophorum*. Norris (33) found an organism resembling *B. necrophorum* in a liver abscess of a man. This organism was associated with anaerobic cocci, the colon bacillus and *Proteus vulgaris*. No pathologic condition of the intestine was reported in either of the liver abscess cases. This does not exclude the possibility that lesions were present in the colon at the time of the entrance of the emboli into the blood stream.

In 1910 Stemen and Shaw (34) described an acute infection of the skin in a patient who was a government meat inspector. While dissecting an ulceration on the lip of a sheep, the patient had scratched his hand on one of the sheep's teeth and subsequently developed an infection of the hand from which *B. necrophorum* was isolated. Shaw (34) isolated *B. necrophorum* in apparently pure culture in pus from a patient with a lung abscess. Cunningham (35) studied two cases which came to autopsy. In one case, *B. necrophorum* was isolated from abscesses and necrotic tissue of the hip joint, lung infarcts, and blood. There was a 15 cm. bluish hemorrhagic ulceration in the lower part of the ileum which was thought to be the portal of entry of the organism. In the other case, it was found in a retropharyngeal abscess with gangrene and extension to the peritracheal and subcutaneous tissue and mediastinum. There were submucous hemorrhages into the ileum. Harris and Brown (36) isolated an organism which they named *Actinomyces pseudonecrophorus* from the uteri of women with puerperal infection. Their strains did not produce spreading necrosis when injected subcutaneously into rabbits.

In 1934 Shaw and Bigger (37) described a case of necrobacillosis of the lung following an upper respiratory infection, in which the organism was found in a surgical specimen in pure culture. Henthorne, Thompson and Beaver (16) found *B. necrophorum* (*Bacteroides funduliformis*) in pure culture from four liver abscesses, three of them from patients with carcinoma

of the rectum. They also isolated it in pure culture from a fecal (?) fistula in a patient with carcinoma of the sigmoid flexure and in mixture with other organisms from a patient with a pulmonary abscess.

No attempt has been made to review all of the reported cases, but rather to point out the various types of lesions in which these organisms are found. In France there has been considerable activity among clinicians and bacteriologists in recognizing these infections, as evidenced by the reviews of Teissier (38), Pham Huu Chi (39), and Lemierre (40). In Germany, Brunner (41) has reviewed the literature, and described three cases of his own in which "*Bacillus funduliformis*" had caused pleural empyemas.

The author has studied strains of *Bacterium necrophorum* from lesions in many parts of the body, such as in chronic ulcerative colitis and cancer of the rectum, iliopsoas abscess, sub-acromial abscess, chronic fistula draining from the breast, and osteomyelitis of femur following middle ear infection. It was also isolated from the blood stream of a child who had a severe angina and developed lung abscesses.

The rôle which *B. necrophorum* plays in chronic ulcerative colitis is not clearly understood. From a group study made over the past eight years the following information has been obtained (26). When the seriously diseased colon is isolated from the fecal stream, as by end ileostomy, aerobic organisms are greatly reduced in number from the colon discharges, the flora becomes almost entirely anaerobic and *B. necrophorum* predominates (23, 24, 43). Such an isolated colon often remains diseased for years with intermittent periods of quiescence and exacerbation. During periods of quiescence *B. necrophorum* usually disappears, only to become plentiful again with each new exacerbation. This organism has been found in the great majority of cases of typical ulcerative colitis when appropriate methods for its detection have been used, but it is not found in the normal colon. It is pathogenic for rabbits, producing in them local abscesses and systemic infection, and also for man as indicated by its isolation in pure culture from liver abscesses, from persistent purulent sinuses, from empyema thoracis, and

from a portal thrombus in a patient who died of ulcerative colitis. Recently a strain was isolated in mixed culture with an anaerobic coccus from a lymph node in the mesocolon in a surgical specimen from a patient upon whom a colectomy was performed. Specific antibodies for this organism have been found in the blood in cases of chronic ulcerative colitis and not in the blood of normal individuals, indicating (43, 30) that the organism is implicated in some way in the mechanism of the disease, either as a cause or as a secondary invader.

From this summary of *Bacterium necrophorum* it is evident that the organism is probably a normal inhabitant of the mucous membranes of man and animals. This is further suggested by the fact that necrotic lesions have been experimentally produced in the colon of monkeys, following which the organism has been isolated (21, 23, 24), whereas they were not found in the normal colon. The fact that *B. necrophorum* has not been found in the normal colon does not indicate that it is not present there, but probably that it is present in insufficient numbers to be detected. The strains in general are not highly pathogenic, although once metastatic abscesses or blood stream invasion occurs the mortality is high. It is important that this bacterium be considered when dealing with pus which is foul smelling or when studying cases of a septic nature where aerobes are not found.

More study needs to be given this group of bacteria, since little is known regarding their metabolism (44). It is desirable that techniques be worked out and put into general use so that the results of various investigators may be adequately compared.

#### *Bacteroides furcosus*

Although McCoy and McClung (2) list 15 references for this organism, a review of these references reveals that some authors were merely repeating Veillon and Zuber's (5) description and showing wherein their strains agree or disagree with the original description. According to Veillon and Zuber, this bacillus is rare and is distinguished principally by its morphology. It appears in pus as a very small rod, terminating in two little branches which give it a Y shape. In culture it forms rods, but

many elements are elongated and divide at one extremity into two branches that end in a swelling or knob; others bear branches which in turn subdivide. The bodies of the bacilli and the ramifications are never very long. The round, or more often pear-shaped swellings are numerous. This bacillus is scarcely larger than *Mycobacterium tuberculosis*, is not motile and is gram-negative. Colonies appear only after 3 or 4 days at 37°C., and not at all at room temperature.

On the surface of agar the colonies are fine, form little gray dots hardly raised above the medium, and remain separate and very small. When magnified they appear as little yellowish masses, transparent at the edges, and very finely granular. Within the agar the colonies are so fine and so transparent that one can scarcely see them; under the microscope they are round, yellowish, with thin, regular edges. They never become large, even when they are well isolated.

In broth the culture forms a fine precipitate. This bacillus does not give off enough gas to make any appreciable bubbles, but it yields a sour, slightly fetid odor. Development is slow but the cultures remain alive 15 to 20 days.

Guinea pigs inoculated under the skin develop abscesses from which they generally recover; some die of cachexia after several weeks.

The foregoing description by Veillon and Zuber (5) does not clearly distinguish these organisms from the *Bacterium necrophorum* group. However, the pear-like swellings in cultures are typical of *Bacteroides furcosus*, and the Y-shaped forms in pus are not characteristic of *B. necrophorum*; but in view of its great pleomorphism it would not be unusual to expect to find such forms in pus. Prévot (3) has named this organism *Ristella furcosa*. Cohen (15) claims to have found it in 2 of 16 specimens from lung abscess. He reports that it produced gas and a fetid odor in Smith-Noguchi medium. His statements are somewhat contradictory regarding dextrose fermentation, since he states:—"Gas was not produced in broth or in dextrose broth. Gas was produced in dextrose, maltose, saccharose and mannite." Lactose and inulin were not fermented. Milk was not coagulated

and gelatin was not liquefied. Aside from the appearance of the organisms in pus, the other properties are not sufficient to distinguish this species from *Bacterium necrophorum*.

*Bacillus fusiformis* (*Fusiformis fusiformis*)

This species is referred to by Prévot under the generic name *Fusiformis* given by Topley and Wilson (14). The literature concerning this organism is well reviewed by Weinberg *et al.* (4).

Isolation of *Bacillus fusiformis* is rather easily accomplished. It is usually found in mixture with many other bacteria, so that it is desirable to streak out adequately on a suitable medium the specimens to be examined. Dilution in fluid media and pour plates involves too much exposure to oxygen for successful isolation. In our experience these organisms may be isolated on 10 per cent sheep-blood agar plates incubated under anaerobic conditions as previously described. Slanetz and Rettger (45) have found that gentian violet at 1:10,000 dilution in 5 per cent blood-agar or 1:20,000 dilution in potato-extract agar permitted good growth of the fusiform bacteria and inhibited heterogeneous types. These workers found that carbon dioxide in an anaerobic environment was satisfactory for good growth of these organisms. This has also been the experience of the author. The colonies are small, and on blood-agar plates a greenish zone of hemolysis may be seen about them.

Morphologically this group of bacteria varies considerably. In lesions, the fusiform bacilli are associated with spirilla. Tunnicliff (46) grew cultures on agar containing ascitic fluid, and in the old cultures found spiral forms. Subsequently (47), she considered the spirilla and fusiform organisms as two phases in the developmental cycle of the same organism. Later (48), she studied the smooth and rough colonies and found the straight forms associated with the smooth colonies, whereas the spiral forms were much more numerous in the rough colonies.

Varney (49) studied 18 cultures from various sources and separated them into four different types based on morphological and serological differences. His types 3 and 4 could be identified

by morphology, whereas types 1 and 2 varied greatly in size and shape and could be differentiated from each other only by serological tests. There seems to be no correlation between Varney's four types and the following.

Slanetz and Rettger (45) have divided these organisms into four groups based on their morphological, cultural and biochemical characteristics. Morphologically they may be distinguished as follows:

"Type I occurs as single cells and in pairs. The ends are definitely pointed. In young cultures they vary in length from  $3-6\mu$ , and in width from  $0.4-0.6\mu$ . They remain fairly uniform in size, even in old cultures, differing from the other types in this respect. They often contain one or two granules. The cells are shorter than those of any of the other types, and they can often be identified by their morphological appearance."

"Type II is long and slender, often growing in long filaments. The ends are definitely pointed, as a rule. The shorter forms vary in length from 6 to  $20\mu$  and in width from 0.3 to  $0.6\mu$ . Numerous granules are present in old cultures."

"Types III is thicker and often longer than type II. Chains can frequently be observed. They measure from  $6-25\mu$  in length and from 0.6 to  $0.8\mu$  in width. The ends are only slightly pointed. In old cultures the cell outline fades away and granules develop."

"Type IV cells are usually larger than those of the other three types. They occur in characteristic chain formation, and it is often difficult to distinguish between individual cells and a chain of cells. They vary from 8 to  $25\mu$  in length, and from 0.7 to  $1.0\mu$  in thickness. Granules appear in old cultures. On agar medium containing 25 per cent carrot extract the cells increase in size, and numerous granules develop after 48 hours incubation. They present an entirely different morphological appearance on this medium than when growing on potato extract agar."

These authors found that types I and IV could usually be identified by morphology, whereas II and III were difficult to separate in this way but could be separated by other tests. Spiral forms were not found in types I, II and III, but a few were formed in type IV which appeared to develop from filaments or from deep staining bodies within the cells, as described by Tun-

nicliff. Colony types could not be distinguished on blood-agar but could on potato-extract agar. They detected no difference in the types from their growth in broth media.

Gelatin is not liquefied by strains of *B. fusiformis*. Slanetz and Rettger (45) studied the fermentation of glucose, sucrose, lactose and mannitol. Their type I and II strains ferment glucose only, type III glucose and sucrose and type IV glucose, sucrose, and lactose; but none ferment mannitol. None produce gas, which is in accord with the results of most investigators, although Prévot (3) reports that very little gas is produced in glucose-agar with serum. The latter also states that mannitol is fermented by most strains. Milk is coagulated but not digested.

Although Rosenow's brain medium is used for the cultivation of many of the non-sporulating anaerobes, in our experience the fusiform bacilli fail to grow in this medium without the addition of blood or serum. A fetid odor is produced when growth occurs.

Although Varney (49) has been successful in separating members of this group by their agglutination reactions, most other workers have failed. In this connection, Slanetz and Rettger (45) make the following statement: "It was frequently difficult to distinguish between specific and spontaneous agglutination. Furthermore, no definite correlation between the agglutination reaction and the type of organism could be established. Most of the strains were either agglutinated by all of the antisera, or did not react definitely with any of them."

*Pathogenicity for man.* Weinberg *et al.* (4) credit Miller (50), rather than Plaut (51) or Vincent (52), as the first to observe fusiform bacilli in ulcerative stomatitis. Since then (1890), many investigators have found this organism in a variety of ulcerative processes. The name of Vincent is frequently applied to one type of infection, namely Vincent's angina. Vincent (52) published a number of papers on this subject. In the first, which appeared in 1896 and was concerned with hospital gangrene, he described *B. fusiformis* as rectilinear or incurving, frequently filamentous, with the extremities pointed and gram-negative. He noticed the formation of granules or vacuoles, the frequency



of involution forms, immotility and absence of spores. In 40 of 47 cases spirilla were associated with the bacilli.

The fusiform bacilli have been found in normal throats and in ulcerative processes involving the mucous membranes of the throat, colon and vagina, also in noma and lung abscesses. In our laboratory we have frequently encountered them in the ulcerated colon.

The interesting question arises as to why stomatitis (trench mouth) and Vincent's angina are not more prevalent. The factors which contribute to natural resistance against these organisms are not understood. Wallace, Wallace and Robertson (53) have shown that daily intravenous injection of 0.25 lethal dose of scillaren B, a squill glucoside, induces a typical clinical picture of Plaut-Vincent's angina in the dog. Typical fusiform bacilli and spirilla were found in smears from the lesions. When the injections were discontinued, some of the dogs recovered.

Lichtenberg, Werner and Lueck (54) found fusospirochetal organisms in about 45 per cent of tonsils removed from 108 children. They observed these organisms in 91 per cent of the membranes that formed over the tonsillar beds after tonsillectomy, and usually in greater numbers than in the tonsils themselves. Sixteen consecutive cases of severe ulcerative stomatitis in children healed in some 4 to 7 days without treatment, which they found to compare favorably with the reports of cases treated with various drugs and other forms of therapy. Recently (55) nicotinic acid has been reported to be a specific therapeutic agent in stomatitis, which would suggest a metabolic disturbance in the host as responsible for infection with these organisms. King (55) inoculated his own mouth with infected material from a severe case of Vincent's disease and failed to induce inflammation or ulceration. The organisms grew in abundance for a short time but disappeared three days after inoculation.

*Bacterium melaninogenicum*

This organism is particularly interesting due to the coal-black appearance of the colonies which develop on anaerobic blood-

agar plates. Prévot (3) has placed this organism in the genus *Ristella*. *Bacterium melaninogenicum*, *Bacteroides melaninogenicus* and *Ristella melaninogenica* are synonyms.

Oliver and Wherry (56) described and named this organism. They cultured it from the mouth, tonsils, infected abdominal wounds, focal infection of the kidneys, the feces, and from the stools of patients with chronic amebic dysentery. Considerable study has been given this organism by Burdon (57), who describes it as a very small, non-spore-bearing, gram-negative anaerobic diplococcobacillus. Its growth in pure culture is feeble, but it grows readily in mixture with other bacteria with which it is found. Mixed anaerobic blood-agar cultures are characterized by a very extensive destruction of hemoglobin, the formation of large amounts of a brownish-black melanin-like pigment, and the production of a foul odor. The pigment develops slowly so that the characteristic black colonies on blood-agar may not be obvious until after 4 or 5 days. The pigment is similar to but not identical with melanin. In culture the organism has marked proteolytic powers, causing rapid digestion of coagulated serum and other native proteins. Burdon (57) questions the purity of the cultures of Oliver and Wherry (56), since the reactions reported by them were similar to those which he obtained in mixed cultures. *B. melaninogenicum* grows in the same colonies with other organisms and is difficult to isolate and maintain in pure culture. Oliver and Wherry (56) did not use plating methods for the isolation of their cultures.

Burdon (57) examined the anaerobic blood-agar slant cultures from 5 cases of uterine infection studied by Schwarz and Dieckmann (9, 10) and identified *B. melaninogenicum* as the pigment-producing organism in them. Schwarz and Dieckmann (9) had found that puerperal fever, (of the type doubtless due to auto-infection), frequently involving the pigment-producing organism, is extremely rare in patients observing good personal hygiene and occurs most commonly in the less cleanly colored ward patients. Whether *Bacterium melaninogenicum* is a true pathogen or a secondary invader is still unknown.

*Bacillus nebulosus*

This organism was described by Hallé (20). It is briefly discussed by Weinberg *et al.* (4) in a chapter entitled "Insufficiently described gram-negative bacilli." It is a small bacillus resembling the bacillus of mouse septicemia (32). Usually straight, it sometimes is curved, appearing as a rod swollen at the center and tapering at the extremities. It is gram-negative, non-sporulating and shows no involution forms. Growth at 37°C. is slow, and no growth is obtained at room temperature. No gas is formed in sugar media. It is inconstant in its pathogenic properties and occasionally produces abscesses in rabbits and guinea pigs.

*Bacterium pneumosintes*

This bacterium has been placed in the Family RISTELLACEAE by Prévot (3) and in a genus *Dialister*, which he defines as including very small, non-motile, gram-negative, non-sporeforming organisms which pass through Berkefeld V and Chamberland L2 filters. Olitsky and Gates (58 to 62) first isolated these organisms from the nasopharyngeal washings of patients in the early stage of influenza. The cultural characteristics are well summarized by Topley and Wilson. This species may be cultured in Smith-Noguchi medium (human ascitic fluid containing a piece of sterile rabbit kidney and covered with a vaseline seal). After 3 to 4 subcultures in this medium, it will grow anaerobically on blood-agar, chocolate agar and Bordet's medium. Morphologically the organisms are minute bodies, arranged singly, in pairs, or short chains; the length varies from 0.15–0.3  $\mu$  and the breadth from one-half to one-third of the length. The center stains more deeply than the ends.

On horse blood agar, the colonies after 7 days' incubation at 37°C. are round, convex, milky-white, opaque, and measure about 0.5 mm. in diameter. They are amorphous with a smooth glistening surface and an entire edge; there is no hemolysis. Following incubation at 37°C. for 5 to 7 days in Smith-Noguchi medium, they remain viable at room temperature for two and a half years. The organisms withstand freezing and drying *in*

*vacuo*, and remain viable for a long time when dried. Organisms in infected rabbit lungs kept in 50 per cent glycerol at 4°C. survive for 9 months, during which time the virulence is maintained. At 56°C. for 30 minutes the organisms in the moist state are destroyed. They pass through Berkefeld N and V filters.

Acid is produced from dextrose; indole, nitrite and catalase are not produced, and methylene blue is not reduced. Agglutinins are formed following injection of cultures into rabbits. Injection of mass cultures intratracheally into rabbits produces a rise of temperature in 24 hours and sometimes a conjunctivitis and a mononuclear leucopenia. Recovery occurs in 2 to 3 days. If the rabbit is killed during the acute stage of the illness, edema and emphysema are found in the lungs. Numerous hemorrhages, discrete or diffuse, are seen on the surface of the lungs; but the pleura is not involved. On section of the lung a frothy blood-stained fluid escapes and hemorrhages are found scattered through the parenchyma. A muco-purulent exudate is present in the trachea and bronchi. These organisms are non-pathogenic to monkeys when injected intratracheally.

Mills, Shibley and Dochez (63) found these gram-negative filter-passing anaerobes in individuals throughout the year, and for that reason consider that no causative rôle can be assigned to them in the etiology of influenza or the common cold.

### *Bacillus ramosus*

This organism is known under several other names, such as *Bacteroides ramosus*, *Fusiformis ramosus* and *Ramibacterium ramosum*. Prévot (3) has placed it in the Family BACTERIACEAE and has given the generic name *Ramibacterium* to rods straight or curved, non-sporulating, non-motile, not ciliated, not encapsulated, gram-positive and presenting pseudo-branching. *B. ramosus* was first described by Veillon and Zuber. A translation of their description follows.

This bacillus is as constant as *B. fragilis* in pus of appendicitis, but it seems less abundant. It appears identical with *B. ramosus* encountered in pulmonary gangrene. In pus it occurs as a small fine rod, not as long as the tubercle bacillus, and is either isolated or grouped in

clusters. In culture, the rods have the same form as in pus, but a large number of them are somewhat larger and especially longer; some assume irregular forms and are straight or concave; the ends are sharp and the thickness is variable; some contain swellings. Some cells are united two by two in a V shape; others are isolated. Certain rods are branched, and a quite long bacillary form may be seen which is divided at one end into two little branches like a V; others bear several of these little branches throughout their length. In some several branches seem to shoot off from one swelling. The bacillus is non-motile, gram-positive, grows only at 37°C., and requires 3 to 4 days' incubation for good growth. On gelatin no growth is observed. In deep agar the colonies are round or oval, granular, brownish-yellow, at first with smooth edges which later appear bristling with very fine short filaments. On the surface of agar the colonies are very small, gray-white and transparent.

Broth is uniformly clouded in 3 or 4 days and forms a muddy, grayish mass. A little gas and a sour fetid odor are given off. Cultures remain viable for about a month; these organisms are non-sporeforming.

When *B. ramosus* is injected into guinea pigs subcutaneous abscesses are formed; in rabbits abscesses are formed and the animals die in 8 to 10 days. Intravenous injection into rabbits causes the death of the animals in several days and gives rise to intoxication and cachexia.

This description by Veillon and Zuber, although incomplete, is very useful in the isolation and identification of *B. ramosus*. Other characteristics of this species are added in the review of the literature by Weinberg *et al.* (4). In peptone-water growth is meagre and indole is not produced, gelatin is not liquefied, and milk is coagulated. Acid is formed in glucose, maltose, galactose, sucrose, mannitol and lactose. No hemolysis occurs on blood-agar.

Weinberg and Prévot grew *B. ramosus* in glucose broth culture for 24 to 48 hours, and then centrifuged the culture. When 3 ml. of the supernatant fluid was injected intramuscularly into guinea pigs, pain was produced in the part injected; dyspnea and respiratory paralysis followed, after which the heart continued to beat for a short time. Upon injection of a sub-lethal dose (1 to 2 ml.) the muscles went into spasm at the site of injection, dyspnea developed and then gradual recovery followed. The toxin is not hemolytic either *in vitro* or *in vivo* and is not precipitated

with ammonium sulfate. Antigenicity was not determined because of the transient nature of the toxin which, together with virulence, was lost after 6 weeks to 2 months of cultivation. Agglutinins were obtained for homologous strains.

*Pathogenicity for man.* *B. ramosus* has been found in numerous infections in man, such as mastoiditis, chronic otitis, pulmonary gangrene, putrid pleurisy, cavernous tuberculosis and gangrenous appendicitis. It is sometimes found in infections of the urinary tract, intestinal ulceration, liver abscess and osteomyelitis.

Lemierre, Reilly and Bloch-Michel (64) have reported five cases of *B. ramosus* infection observed at the Claude-Bernard Hospital, Paris. The first was one of gas gangrene due to *B. ramosus* and aerobic hemolytic streptococci following a hypodermic injection. The patient recovered after incision and drainage of the wound. In the four other patients *B. ramosus* was isolated from the blood. In one of the latter patients the fact that the blood-culture was positive only once out of five times raises the question as to whether the bacillus might have been only a transitory invader. In the three others, whose blood-cultures were positive, the authors were inclined to believe that the organism was a secondary invader. Compared to the gravity of infections due to *Bacterium necrophorum*, those due to *B. ramosus* are relatively benign.

### *Bacteroides serpens*

This organism has been classed in the Family RISTELLACEAE and in a genus *Zuberella* by Prévot (3), who defines the genus as containing: Straight rods, non-sporulating, gram-negative, motile, ciliated and not encapsulated.

This organism is known under the names *Bacillus serpens*, *Bacillus radiiformis* and *Zuberella serpens*. The species was first described by Veillon and Zuber (5) as a small rod, quite bulky, regular, with rounded ends. In cultures, the cells are often united two by two or form pseudo-filaments. It is slightly motile and progresses especially by undulation. It develops between 20° and 37°C. Gelatin is liquefied. In agar at 37°C. at the end of 24 hours little colonies appear. When magnified,

they look like little round masses, clear, grayish, granular and shaded, and sometimes a bunch of threads appears at one of the poles. Later the colony, growing larger, becomes more opaque and the edges more clearly defined.

On the surface of anaerobic plates little dots appear which are scarcely visible at the end of 48 hours; later the colonies form little cloudy masses which are transparent. Broth becomes very turbid during growth and then clears, leaving a white sediment in the bottom of the tube. The cultures give off a fetid odor but deep agar is not broken. The cultures remain viable for 20 to 25 days. *Bacteroides serpens* is gram-negative and strictly anaerobic.

The cultures are pathogenic to the mouse, guinea pig, and especially the rabbit. Inoculated under the skin, they produce abscesses, and the animals die of cachexia at the end of 7 to 8 days. Pus containing this organism in mixture with others is more virulent than the pure cultures alone. Veillon and Zuber (5) obtained their culture of *Bacteroides serpens* in mixture with *B. ramosus* from a child with a mastoiditis, who was operated upon and died 24 hours later. At autopsy an otitis media, gangrenous abscess in the sphenoidal lobe and gangrenous foci in two lobes of the lungs were found. The abscesses in the lungs and the one in the brain contained foul-smelling pus.

Prévot (3) lists the following additional characteristics of *Bacteroides serpens*. Clouding occurs in peptone-water. No indole is produced. Milk is acidified, then coagulated and gas is given off. Brain medium is blackened. Acid and gas are produced in glucose, levulose, maltose, galactose and lactose broths. No toxin or hemolysin has been demonstrated.

#### DISCUSSION

In this review only the non-sporulating anaerobic bacteria of medical importance have been considered. Those of non-medical importance are little known, even though in the intestinal tract they may outnumber *Escherichia coli* (14). There are many reasons why our knowledge of this group of anaerobes is so meager, although the organisms have been encountered in a wide

variety of lesions. In the first place, they have been assigned names and characteristics on the basis of inadequate study. Furthermore, medical bacteriologists know practically nothing about the group, and their attempts to inform themselves have led more to confusion than to systematic knowledge. In this review only those organisms which have been most studied are considered, and of 266 species only 4 members of the coccus group (*Streptococcus anaerobius*, *S. foetidus*, *S. putridus* and *Staphylococcus parvulus*) and ten of the rod-shaped organisms were selected from the bibliography of McCoy and McClung (2). Of the 10 members of the bacterium group two may be the same species (*Bacterium necrophorum* and *Bacillus funduliformis*) and of the remaining eight only four have been studied to any extent (*Bacteroides fragilis*, *B. fusiformis*, *Bacterium pneumosintes* and *Bacillus ramosus*). Not more than 18 references have been listed (2) for any of the remainder (*Bacteroides furcosus*, *Bacterium melaninogenicum*, *Bacillus nebulosus* and *Bacteroides serpens*).

One characteristic of all of these organisms is that they are associated with ulcerative processes involving the mucous membranes and that under certain circumstances they may invade the tissues and produce abscesses from which foul-smelling pus is obtained. They are frequently found in the blood stream in septicemias. An effort should be made on the part of teachers in medical schools to inform students of this group of organisms and to teach those in laboratories of medical bacteriology to be aware of and to recognize them. The author apologizes for using the assortment of generic names commonly applied to these bacteria and listed in the subject bibliography of McCoy and McClung. It would seem better to use the generic name *Bacterium* for the non-sporeforming rod-shaped species until such a time as a suitable classification may be given. Prévot is to be complimented on his attempt to classify them. The difficulty with his classification is that he has accepted inadequate descriptions of organisms and thus increased the number of species. A careful study of this group of bacteria, using uniform methods, would eliminate the species which were created on the basis of inadequate study.



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# THE CHEMISTRY AND SEROLOGY OF THE VIBRIOS

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Philosophical ideas about disease causation, which at the end of the 18th Century had largely replaced the notions of a *contagium vivum*, received a rude shock with the first European outbreak of cholera in 1826. Thereafter cholera shared with anthrax the honor of stimulating much of the interest and research which culminated in establishing the bacterial theory of disease in the late 19th Century, and of becoming, as Beard (10) has stated, the world's most effective teacher of sanitation and public health. In more recent years, the study of cholera, like that of other diseases, has varied in interest and importance from time to time. During the last decade cholera research has gone through one of its more active periods. Extensive programs have been carried out in India, the Far East and Europe on many aspects both practical and theoretical, under the auspices of the Indian Research Fund Association and of the *Office International d'Hygiene Publique* in Paris, as well as under those of other bodies. Antigenic and chemical structure, variation, metabolism and the renewed study of the El Tor strains since their rediscovery at El Tor and in the Dutch East Indies, have all been the subject of informative research which it is proposed to review in this paper. It has been found necessary to omit consideration of the extensive reports on the experimental and therapeutic uses of bacteriophage, the epidemiology of cholera except as it is related to the source of some of the strains discussed, the production of cholera vaccine and its use in the field.

The main emphasis in recent cholera research has been to differentiate between pathogenic and non-pathogenic forms. The

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present idea is that the authentic cholera organism possesses the following combination of characteristics: fermentation of mannose and sucrose but not of arabinose; failure to hemolyze goat's blood; and agglutination with O-group I serum according to Gardner and Venkatraman (33) (see Table 3). As will be shown in the ensuing discussion, it is the combination of these characteristics which alone defines the authentic vibrio and none of them will do so by itself. This fact is, in the writer's view, difficult to reconcile with the orthodox teaching that the cause of cholera is a distinct entity utterly unrelated to other vibrios. The study of variation together with chemical data to be discussed below will indicate that the whole vibrio group is closely interrelated.

#### THE CHEMICAL STRUCTURE OF THE VIBRIOS

*Carbohydrates.* Landsteiner and Levine (62) extracted a substance having the characteristics of a polysaccharide from a vibrio strain. Fractions were extracted by hot 75 per cent alcohol which were antigenic, gave tests for protein and polysaccharide, and appeared to be formed from a combination of these two substances. Upon hydrolysis they showed strong reducing powers and yielded osazones. By dissolving one of the fractions in dilute alkali and reprecipitating with alcohol, another fraction was obtained which gave only faint protein reactions and an intense Molisch reaction; it was almost entirely non-antigenic, was not a protein, contained N and P, and when dissolved in acid solution and hydrolyzed gave rise to a substance of acid character. These findings have been entirely confirmed by later workers.

Linton (63) extracted a crude polysaccharide fraction by boiling vibrios from different sources with N/20 acetic acid until the suspension coagulated. The supernatant was worked up to free it from protein and the final material, which was biuret-negative and gave a Molisch test at a dilution of one million times, was used as antigen in precipitin tests. The conclusion was reached that agglutinating, non-agglutinating and water vibrios contained a common or closely related carbohydrate fraction

which differed from similarly prepared fractions of dysentery and typhoid organisms. Further work was then undertaken by Linton and his collaborators (80 to 83, 85) to see if specific polysaccharides could be extracted; and preparations by more exact methods were made from smooth and rough cholera vibrios and from water vibrios. This extensive chemical work led to the conclusion that at least two structurally different polysaccharides were present in the vibrio group as a whole. Each strain contained an aldobionic acid made up of galactose and glucuronic acid; in each a second readily hydrolyzable sugar was present as well; in some of the strains this sugar was galactose and in others it was arabinose. The galactose- and arabinose-containing carbohydrates were also identified in the "rice-water" stools of patients with cholera (84). By chemical methods similar to those used for isolation from vibrio cultures, biuret-negative substances were obtained from stools which after hydrolysis yielded reducing substances, and gave characteristic phenylosazones for galactose in seven cases, and for arabinose in three. At the same time it was shown (83) that polysaccharides from vibrios neutralized the antibacterial effect of immune sera.

Later studies by the same group of workers revealed a third type of polysaccharide in the vibrio group. In these strains, no aldobionic acid could be found in the hydrolysate in spite of repeated attempts and the use of various methods, and glucose was the only simple sugar identified, although the presence of non-amino nitrogen, amino nitrogen and phosphorus indicated a complex structure. The glucose type of polysaccharide was found in a strain which had dissociated from another strain having the galactose type of polysaccharide and this observation was the first which led to the study of the chemical basis for variation. The glucose-containing type of polysaccharide had been discovered previously by Jermoljewa and Bujanowskaja (52) in an old laboratory strain of *Vibrio cholerae*. When the study of the three polysaccharides had been carried thus far, a shorter method for identification was developed (72) in which a suspension of the whole organisms was directly hydrolyzed in dilute acid. The



manipulations necessary to obtain a phenylosazone were then carried out and comparative tests on a dozen strains indicated that the same monosaccharide was separated out whether the polysaccharide was first separated from the organisms, purified and then hydrolyzed, or was obtained directly by the hydrolysis of the whole organisms. This new method permitted the rapid identification of the characteristic polysaccharide.

White (159) had reported that a complex of polysaccharides with different ranges of serological reactivity existed in the vibrios. Since chemical work had indicated the presence of but a single polysaccharide in each vibrio, it was of interest to study this discrepancy along the lines taken by Avery and Goebel (6) when a somewhat similar situation had arisen in the study of pneumococcus. Linton and Mitra (68) showed that all three types of vibrio polysaccharide existed in the cell as acetyl compounds. Acetylation was of course readily destroyed by the usual method of extraction in which alkali was used. The relation of this finding to White's serological complex will be discussed later. The acetylated and deacetylated forms of each polysaccharide differed in their specific rotations from each other and from each of the two forms in the other two types. Type I (galactose) polysaccharide was almost completely extractable from the cell in the acetylated form; while with the Type II (arabinose) and Type III (glucose) a considerable proportion was not extractable in this form but could be subsequently removed in the deacetylated form by the use of dilute alkali. The possibility of contamination of these polysaccharides by agar was checked by preparing them from peptone water cultures and arriving at identical results (68). The highest degree of purity in the preparation of a vibrio polysaccharide was attained by Shrivastava and Seal (130), who based their method upon that of Heidelberger, Kendall and Scherp (47). Their product was a white stringy material resembling fibers of dried filter paper. It was difficultly soluble in water and gave a solution of high viscosity at concentrations of less than 3 mg. per ml. Its nitrogen content was 2.62 per cent and it had a specific rotation of  $+58.0^\circ$ , and a positive Molisch test at 1:6 million dilution. During

hydrolysis a sudden rise in reducing power occurred between the 3rd and 4th hours, and during the same period the precipitin reaction with antisera against the whole organisms abruptly disappeared. This sudden rise appeared to indicate the presence of a second substance in the solution, whose rate of hydrolysis differed from that which hydrolyzed first, and recalled the hydrolysates in which an aldobionic acid was present (81). In spite of all attempts, however, Shrivastava and Seal could not isolate reducing substances from it, other than glucose, thus confirming earlier work on this type of polysaccharide (66, 86). The product was reactive in the precipitin test to 1:12 million dilution, and the reaction was specific for organisms having the same polysaccharide. Using the same chemical methods, Linton, Shrivastava and Seal (87) studied the character of the polysaccharide which a single strain produced when grown on eight different media, containing various combinations of peptone, infusion broth, buffers and glucose. The result was to show that the type of medium had a profound effect upon the specific polysaccharide, variation from 1.8 to 16.4 mg. per liter and of precipitin titres from  $2.4 \times 10^3$  to  $1.6 \times 10^7$  being observed. The less reactive polysaccharides were amorphous when dry and gave clear solutions of no obvious viscosity while those with high titres were like the polysaccharide described by Shrivastava and Seal. It was suggested that the physical appearance and characteristics as well as their serological reactivity was a function of the molecular size. The best medium consisted of 1 per cent peptone in infusion broth; the presence of buffers and especially of glucose in the medium gave lower yields and less reactive products. Both volatile and non-volatile organic acids were present after short periods of growth in sugar-containing media and these acids may have been responsible for the lower yields and lessened serological reactivity. It was also observed that older strains seemed to be less active producers of polysaccharides than those more recently isolated.

In connection with the purification of polysaccharides of the different types, Linton and his co-workers (88) found that they varied in specific rotation, percentage of acetylation, maximum

yield of reducing substances, ash and nitrogen, but there did not appear to be any constant differences which could be used to mark off one from the other, except of course the isolation and identification of the type sugar. They found that polysaccharides of case strains isolated from the first part of an epidemic developed a heavy opalescence when their aqueous solutions were treated with sulfuric acid, while strains isolated late in an epidemic, as well as water, carrier and old laboratory strains, did not do so. Study of the opalescent material indicated the probability that it was a carbohydrate-phospholipid complex. Checcacci (21) has recently reported the isolation from vibrios of a similar complex, which was antigenic and appeared to represent the O-antigen. The highly purified polysaccharides of Linton and Shrivastava and Seal were not found to be antigenic (130, 88). These were used in a series of cross-precipitin tests, and while there was a general agreement between chemical structures of the polysaccharides and their precipitin cross-reactions, the exceptions and discrepancies which were observed made the test useless in practice. It was considered possible that the degree of purification reached in the polysaccharides might have removed some factor essential for complete specificity.

Much information about vibrio polysaccharides, especially in their serological relations, has been obtained through the work of White. Yang and White (169) prepared biuret-negative and Molisch-positive substances by methods which White had previously used in his studies on *Salmonella* (153). When derived from smooth vibrio colonies, these substances absorbed 90 to 98 per cent of the O agglutinins from smooth antisera; but substances derived from rough strains by similar methods did not reduce the agglutinins from the same antisera. On the other hand, rough antisera had their agglutinins completely removed by both rough and smooth substances. This relationship is quite parallel to that found in R and S races of other bacteria (41). The R and S substances also gave a certain amount of specificity in cross-precipitin reactions with their homologous and heterologous sera. In further work, White (160) showed that the A-type phage was specifically inhibited by the poly-

saccharide substance of true cholera and El Tor vibrios. In this instance he prepared the polysaccharide substance by digesting the vibrio suspension with papain, precipitating the extracted material with alcohol and purifying the extract with picric acid and repeated precipitations with alcohol. Polysaccharides derived from non-choleric vibrios and from rough and "rho" races, did not inhibit the A-type phage. This finding indicated that in the vibrios the smooth type polysaccharide bound the phage and led further to the conclusion that the resistance of the secondary cultures resided in the loss of this substance rather than in any positive modification of the vibrios. White also noted that some of the several types of phage were inhibited by the acetone-ether soluble part of the vibrios. Pandit, Maitra and Datta Roy (110) also studied the inhibiting effect of vibrio extracts upon phage, but they did not obtain any very definite correlation between the resistance of a strain to a phage and the inhibition of the phage by an extract of the strain, although in general the more phages a strain resisted, the fewer phage types its extract would inhibit. Gough and Burnet, who had first developed the method, demonstrated that the inhibiting agent of their extracts was a polysaccharide (38). It was not evident, however, that Pandit, Maitra and Datta Roy had actually extracted a polysaccharide for use in their tests, and hence their failure to obtain more definite results was perhaps not surprising. The extracts were classified into three groups according to their phage-type inhibition, but these groups were only partially similar to the groups found by chemical analysis of the polysaccharides. In an extension of this work, Maitra (93) was unable to show any correlation between the phage-inhibiting extracts and either Linton's classification of the vibrios or the serological classifications with O-antigens. Again it was not clear from the data presented just what portion of the vibrio was being used as the inhibiting agent, but the assumption was made that it was a polysaccharide complex.

In summarizing his views at the time, White (159) concluded that each type of vibrio growth (S, R and rho) had its own characteristic complex of polysaccharides, the types differing from

one another by the loss of one member of the complex. The specific carbohydrates were four in number in the S form ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ), three in the R form ( $\beta$ ,  $\gamma$ ,  $\delta$ ), and two in the rho form ( $\gamma$ ,  $\delta$ ). Type  $\alpha$  was dominant in the smooth form,  $\beta$  in the rough, and  $\delta$  in the rho, and the loss of these dominant substances from the S and R forms led to the domination by another of the substances, which was previously present but masked. Chemical information about these fractions was scant, and White stated that referring to them as polysaccharides might be inaccurate, although his hypothesis assumed them to be such. All were biuret-negative, Molisch-positive substances which yielded reducing substances on hydrolysis. The crude fractions I and II had 3 to 4.5 per cent of nitrogen, irrespective of the type of strain from which they had been derived. The  $\alpha$  substance was altered when treated with alkali, while the others were resistant. Precipitin tests were carried out not with the separated fractions, with the exception of  $\alpha$  in one case, but with the crude fractions I and II; and from the S, R, and rho forms in each case. The results led White to the conclusion that his hypothesis of the distribution of this complex in the vibrios was correct, although he was admittedly working with impure fractions about whose composition little was known. The amount of evidence given in the paper does not seem adequate to support the rather complex interpretation of carbohydrate structure which it is made to bear. He emphasized the statement that more work was being undertaken upon this carbohydrate-complex, but there is no mention of it in any of his subsequent publications.

In a further contribution to the problem, White (162) noted that antisera prepared by injecting Inaba and Ogawa type vaccines (table 3) into rabbits varied greatly between high type-specificity in which they acted very selectively on the homologous type, and group specificity in which they acted to titre on other cholerigenic vibrios, although not appreciably upon vibrios from other sources. The difference appeared to lie entirely in the rabbit chosen for injection. He stated that these facts complicated the use of standard vaccines for the preparation of agglutinating antisera. In the hope that rabbits might respond

more uniformly to fractions than to whole vibrios, White injected "protein-free" specific substance. This material proved to be actively antigenic, but the same variation in specificity was obtained as with whole vibrios. It is worth noting in this connection that other attempts (Shrivastava and Seal, 130) to produce antibodies by the injection of highly purified polysaccharide were entirely unsuccessful, leading one to suppose that White's fractions were still contaminated with protein.

In view of the finding by Linton and Mitra (68) that the vibrio polysaccharides were acetylated and that treatment with alkali caused them to lose their acetyl groups, White suggested in the paper under consideration that the change in reactivity which he had previously attributed to the disappearance of the S polysaccharide after treatment with alkali was actually due to the removal of acetyl groups. If this were the case, the R polysaccharide would not be a separate entity "present but masked," in the smooth vibrio, but a deacetylated or non-acetylated smooth polysaccharide. But it would also follow that the smooth forms produced acetylated polysaccharide while in the rough ones non-acetylated polysaccharide would be found, and for this idea there is no evidence one way or the other since Linton and his co-workers unfortunately did not study acetylation in relation to smoothness and roughness, although they did find that the amount of acetylation appeared to vary a good deal from strain to strain (87, 88). White's idea of the smooth polysaccharide was that "it possesses a number of receptor groupings, some of which are type specific, some group specific, some alkali labile, some resisting alkali, and that in the rabbit now one, now another of these receptors plays the dominant rôle in stimulating antibodies." No view was expressed about the relation of this complex to that which he had described previously (159).

Two brief papers have concerned themselves with the preparation of residual antigens from the vibrios by the method of Boivin and Mesrobian (19). Damboviceanu (27) sought to find if true *V. cholerae* and non-agglutinating organisms from various sources differed in respect to the extracted portions. The specificity and range of these antigens in the precipitin reaction

was exactly the same as that of the whole organism in the agglutination test. Damboviceanu refrained from concluding, however, that in view of his results the fractions were the "cause" of the specificity. Reynal, Lieou and Feisolle (116) applied the same method and obtained an extract which in addition to being specifically precipitated by homologous antiserum, was also toxic, antigenic and caused anaphylactic shock when injected into guinea pigs previously sensitized with whole organisms.

*Proteins.* The chemistry and serology of the vibrio proteins have formed the subject of several papers. The methods used by Linton, Mitra and Shrivastava (77, 67) depended essentially on the observations of Kossel and Weiss (57) and Dakin (22) that proteins dissolved in weak alkaline solutions gradually altered their optical properties. This change took the form of a diminution in rotatory power, which was rapid at first but gradually lessened until after some days a constant value was reached. Lloyd (53) has discussed the chemical basis of this change. In the earlier use of the method of Kossel and Dakin, the proteins were allowed to stand in alkali until their rotation had become constant. They were then hydrolyzed by acid, the resulting amino acids isolated, and their optical properties determined. With the exception of glycine, the amino acids are ordinarily optically active after acid hydrolysis. The amino acids from the alkali-treated proteins were found to vary in this respect, some being active, some inactive, and some partially active. If the same amino acids in two proteins were found to have different optical properties after alkali treatment, it was assumed that they could not have occupied similar positions in the original proteins, since they had been acted upon differently by the alkali. A number of proteins were studied in this way by the earlier workers; the method was found to be dependable but technically not entirely satisfactory. The isolation and purification of the individual amino acids was a lengthy process and required much material. Woodman (168) showed that a simpler method would yield the same result. He followed the optical activity of the protein solution while it was dissolved in alkali, and plotted the specific rotation against time. The curves obtained in this way

were perfectly smooth and had concordant shapes in duplicate experiments.

Linton, Mitra and Shrivastava (77, 67) applied the method to globulin solutions obtained from a variety of vibrios. Altogether about 200 vibrios were studied and in the whole group only two kinds of curve were obtained. Data representative of both are shown in table 1. Carrying the reading up to 15 days gave no further changes. Proteins giving curves similar to 2027 were designated Protein I, while the other curve was called that of Protein II. With either kind of protein the agreement in the curves from one strain to another was extremely close, and the

TABLE 1

*Specific rotations of the pseudoglobulins of cholera vibrio 2027 and water vibrio W3075 in N/2 NaOH*

HOURS	CHOLERA VIBRIO 2027 SPECIFIC ROTATION	WATER VIBRIO W3075 SPECIFIC ROTATION
1	-76°	-71°
5	-65°	-60°
24	-44°	-39°
48	-36°	-31°
96	-27°	-22°
120	-25°	-20°
145	-23°	-18°
196	-20°	-15°
217	-19°	-14°
264	-19°	-14°

readings did not vary more than a degree and were usually identical at the same time period.

Mitra (98, 99) undertook to study the two kinds of protein by examining the optical properties of the amino acids themselves after they had been released by acid hydrolysis from the racemized material. The diamino acids were prepared by the method of Kossel and Kutcher (56), and the melting points of the acids were in all cases similar to those found by other workers and the percentage of nitrogen was close to the theoretical. When the specific rotations of the diamino acids from the normal and alkali-treated proteins were compared it was found that histidine was optically inactive in both, while lysine was optically



active. On the other hand, arginine was partially active in Protein I and totally inactive in Protein II. This finding was taken to indicate that lysine and histidine occupied similar positions in the two proteins, while it was assumed that the position of arginine differed. Mitra concluded that structural differences did exist between the two protein types in respect to the configuration of arginine. Using the isobutyl alcohol method of Dakin (23 to 25), Mitra then investigated the monoamino acids in normal and alkali-treated proteins (99). Glycine was optically inactive, and the other amino acids which were optically inactive in both Protein I and Protein II were alanine, valine, tyrosine and aspartic acid. Leucine was optically inactive in Protein I, optically active in Protein II; glutamic acid active only in Protein I; proline partially active in Protein I and less active in Protein II; while hydroxyproline was optically active in the case of both proteins. Isoleucine, phenyl-alanine, serine and cystine were not found in either protein, probably because they were present in too small amount to allow their isolation. Mitra's general conclusion was that glutamic acid and leucine differed completely in their relative positions in the two proteins, while arginine and proline were also somewhat differently placed.

Mitra also studied the purified globulins of the two types of protein by means of the spectrograph (101), and observed that each yielded a concordant curve (in three specimens), which differed from the curve of the other protein type. The curves were distinct throughout the course of the experiment. The result was the same as that from racemization in indicating the existence of two proteins in the vibrio group.

Other studies have been made on protein fractions of vibrios, in contrast with the whole proteins just described. Linton and Mitra (65, 71) extracted a protein which they designated as fraction A by treatment with 0.025 N HCl-absolute alcohol. It had a specific rotation of about  $-12.0^\circ$  and a nitrogen distribution showing about twice the amount of amide nitrogen and half the amount of humin nitrogen as the whole protein. The A-fraction occurred in the globulin fractions of the protein, and it was destroyed when the above extraction was attempted

in 0.125 N HCl-absolute alcohol. The yield was about 1 to 2 per cent of the dry weight of the organisms. A second protein fraction, designated B, was also obtained but it did not differ significantly from the whole protein in its nitrogen distribution. Fraction A appeared to be chemically the same irrespective of the type of vibrio from which it was derived, and while it represented only a small part of the whole vibrio, it approached the latter in its serological activity and was as effective an antigen. It was also able to prepare the skin for reaction in the Shwartzman phenomenon (89), and it contained much of the vibrio polysaccharide. Since fraction A appeared to contain much of the serological reactivity of the vibrio, it was assumed that it represented the outermost portions of its surface, and the mode of extraction pointed in the same direction.

Similar acid alcohol soluble fractions were obtained by White (154). His extract was divided into two parts,  $Q_1$  and  $Q_2$ , on the basis of differential solubility in dilute acid and alkali. Both were active antigens, giving rise to sera which agglutinated all types of vibrios, but which did not precipitate the soluble polysaccharides of S and R vibrios. Further work (157) showed that the  $Q_1$  proteins from S and R strains were serologically the same. Anti- $Q_1$  sera agglutinated most living smooth vibrios only at a low dilution, while R strains were slightly more sensitive. On heating the vibrios at  $100^\circ$  for a few minutes, most of them became highly agglutinable with all  $Q_1$  antisera. In accordance with his hypothesis of bacterial structure, White concluded that  $Q_1$  substance was present in only a trace on the surface of living vibrios, but was more fully exposed and hence more reactive after heating. He also discovered one strain which was highly agglutinable with  $Q_1$  antisera while alive, and concluded therefrom that  $Q_1$  was not an artefact but was present as such in the living organism.  $Q_2$  antisera were somewhat more specific for true cholera vibrios and they also showed the generalized reaction with heated vibrios which was found with  $Q_1$  antisera. White indicated the possibility that the Q substances contributed to the non-specific O agglutination of heated vibrios, discovered by Gardner and Venkatraman (33). In addition, White (165) obtained antigenic

protein fractions whose antisera reacted with similar antigens from a wide variety of vibrios. These antisera did not agglutinate whole vibrios and the fractions were accordingly parts of the deeper structure of the organism, according to his hypothesis of bacterial structure. The use of NaOH in their preparation would also render it a distinct possibility that they were artefacts.

Linton, Mitra and Shrivastava (76) analyzed the protein portions of agglutinating and non-agglutinating vibrios by the van Slyke method and showed that they formed a homogeneous group in their nitrogen distribution. In comparison with the figures collected by Hirsch (50) for *Corynebacterium diphtheriae*, *Escherichia coli*, *Mycobacterium tuberculosis* and a strain of nitro-bacteria, *Vibrio cholerae* had a relatively simple structure, which was marked off in its nitrogen distribution from that of other bacteria. The cholera vibrios had a relatively high content of the simpler amino acids and their basic amino acid content was definitely low, while the smallness of the amide nitrogen figure pointed to a comparative simplicity in their protein organization. Mitra (100) isolated the nucleic acid from vibrio protein and found both cytosine and uracil, while thymine was absent, and concluded that the acid had the pyrimidine constitution of a plant and not of an animal nucleic acid.

The elementary constituents of the vibrios were studied by Linton, Shrivastava and Mitra (85), who found that these organisms did not differ significantly among themselves; all contained about 0.5 per cent of phosphorus and slightly more sulphur, while the fat content of 2.5 per cent was that usually found in bacteria. Carbon, hydrogen and nitrogen showed the usual values for normal proteins. Damboviceanu and Barber (26) had shown that different kinds of bacteria grown under identical conditions differed both in the weight and in the composition of their ash. Continuing this work with vibrios, Barber (9) noted variations from 3.9 to 13.5 per cent in amount of ash present after growth under identical conditions. Strains having the smaller amounts of ash had the most calcium salts, while those having most ash had most salts of potassium, sodium and phosphorus. The vibrios were found to be poorer in calcium

salts than other microorganisms. Barber also showed that vibrio strains having the most ash are also those most strongly agglutinated by acids and tryptoflavine and having the highest surface potential; and in fact throughout her work there is the suggestion that the rough forms have more ash than the smooth. Her further observation that any strain might vary greatly in ash content from time to time in spite of the use of the same culture medium, might also find its explanation in the S-R transition, although Barber did not specifically study this change. Further evidence for the effect of dissociation upon ash content is found in the observation of Damboviceanu and Vasilescu (28) that bacteriophage derivatives, that is, rough forms, had a richer content of ash than the parent smooth strains.

TABLE 2  
*A chemical grouping of the vibrios*

GROUP	PROTEIN TYPE	POLYSACCHARIDE TYPE
I	I	I
II	I	II
III	II	II
IV	II	I
V	II	III
VI	I	III

*A classification of the vibrios based upon their chemical structure.* Linton (64) outlined a classification of the vibrios based upon their protein and polysaccharide structures. Using the methods which have been reviewed, it was found that one polysaccharide and one protein was commonly obtainable from each strain of vibrio; when exceptions occurred it was invariably noted that the strain was undergoing dissociation. The study of numerous strains from all parts of India and from other places as far apart as Cairo, Tokyo, Basrah and Shanghai over a period of several years did not reveal the presence in them of any chemical structures other than those already described. Given a single protein and polysaccharide in each vibrio, it was possible to divide the strains into six groups, which were numbered in the order of their discovery as shown in table 2.

The first three groups were formed when the existence of two of the polysaccharides and the two proteins had been established. It was then possible to predict that a fourth group probably existed and when El Tor strains were studied they were found to have the expected structure (Linton, Mitra and Shrivastava, 77). Group V was formed when the glucose-containing polysaccharide was discovered and the existence of yet a sixth group, which would contain Protein I, was then predicted (Linton and Mitra, 67). In the study of the Japanese type strains the expected combination of constituents was found (Linton, Shrivastava and Mitra, 86). The protein-polysaccharide complex which forms the bacterial body can be broken down in many ways, limited only by the patience of the investigator and the number of methods and solvents he is prepared to use. In the vibrios, it appeared preferable to consider the protein and polysaccharide of each strain as a unit, and the resulting classification was far simpler than those obtained by purely serological methods, as will be shown in work to be reviewed later.

The classification shows that the vibrios form a closely allied group of organisms with interrelated chemical structures shared in an interesting and probably significant way. The strains of Groups I and II possess the same protein and different polysaccharides. These strains are derived from cases of cholera and have the serological and biochemical characteristics of the authentic O-group I *Vibrio cholerae*. Group I strains are far more common than those of Group II, which have, however, been isolated from epidemics with a high mortality in Assam. The phospholipoid fraction is common to both types when isolated in the early part of an epidemic (Linton, Shrivastava, Seal and Mookerji, 88), but it is not found in strains of other groups. The harmless water vibrios, which are so heterogeneous serologically (Taylor and Ahuja, 142), form a single chemical group with a homogeneous structure. They fall into Group III, which differs in its protein structure from the authentic cholera vibrios, and resembles Group II in its polysaccharide. The vibrios of Group IV, which came from El Tor and from chronic vibrio carriers in India, are believed on epidemiological grounds to be

harmless, although serologically the most refined methods have so far failed to distinguish them from the cholorigenic vibrios. The recent finding of hemolytic strains resembling El Tor strains in an epidemic in Celebes has once more cast doubt on the status of these strains in cholera epidemiology (de Moor, 102, 103). As will be pointed out later, when strains of Group I change their chemical structure, they usually vary to Group IV, and this may be a fact of some importance. Unfortunately, the Celebes strains have not yet been chemically classified. Group V, which, like III and IV, contains protein II, consists, like Group IV, of strains from chronic vibrio carriers. In cholera epidemiology a sharp distinction has been made between contact and chronic carriers. The latter carry vibrios which appear non-cholorigenic and these are generally of Group V. By contact carriers are meant those who have acquired vibrios from being in recent close contact with a case of cholera. The strains isolated from them are, as one would expect, of Groups I and II. Group VI strains are only rarely isolated in nature and representatives of this group are generally found among collections of old laboratory strains. They appear to be the result of polysaccharide variation from Group I after long-continued growth on artificial media.

#### SOME PRODUCTS OF VIBRIO GROWTH

Hirsch (48, 49) studied the metabolic activities of the vibrios. Under aerobic conditions, the metabolism of these organisms did not differ significantly from that of other intestinal organisms. Atmospheric oxygen acted as a hydrogen acceptor in the deamination of amino acids, and ammonia, acetic acid and carbon dioxide were formed. In the presence of oxygen and glucose, aspartic acid acted only as a source of nitrogen, and about 20 per cent of the sugar was oxidized while about 80 per cent was fermented; the vibrio appeared to prefer fermentation as a source of energy even under atmospheric oxygen tension, just as it preferred to use carbohydrate rather than protein generally in its metabolic activities. Under anaerobic conditions, vibrios could not grow in the presence of protein alone; when the oxygen

tension was that of the intestinal tract, carbohydrate was essential for growth. Under these conditions, glucose was broken down in two distinct ways, first to form lactic acid, and second to form acetic and formic acids and small amounts of ethyl alcohol. These studies by Hirsch laid a rational basis for the production of toxin by *V. cholerae*, to be described below.

Of considerable interest are the attempts which have been made to classify the vibrios on the basis of their acid production in various sugars. In the hands of Heiberg and Taylor this study has yielded valuable information regarding the interrelations of the vibrio groups. Heiberg first showed (45) that acid production in media containing mannose, arabinose and sucrose defined six vibrio groups. His first type, which produced acid from mannose and sucrose but not from arabinose, contained all vibrios of the serological type of O-group I of Gardner and Venkatraman (33), which are considered the true cholorigenic vibrios (table 3). He worked with 384 vibrios from varied sources, and of these 287 fell into the first group and 75 into the second, while the remaining four groups each contained only a few strains. Combiesco-Popesco and Cocioba (20) classified 107 strains from different sources into agglutinable and non-agglutinable types on the basis of their reaction with O-group I antiserum. They found that all the agglutinable vibrios fell into Heiberg's Group I; inagglutinable vibrios derived from cases of clinical cholera into his first two groups; while the El Tor strains of the 1930 pilgrimage were scattered throughout the first four groups. It was apparent that some discrepancies existed between O-group I agglutinability and group I fermentation, and the evidence that the purely biochemical classification of Heiberg would alone not lead to strict accuracy in diagnosis was completed by the work of Taylor, Read and Pandit in India (145). They found that 117 case strains and eight carrier strains, all O-group I agglutinable, fell into Heiberg's first group. This set of 125 strains had been freshly isolated in field studies. On the other hand, over a quarter of the inagglutinable strains from human sources gave the same reaction, and 11 per cent of the water vibrios as well showed the fermentation reaction of Type

I. This result was confirmed by Mertens and Mochtar in Java (97). It was clear that the biochemical information was less exact than that furnished by serological methods, but at the same time it was evident that where large numbers of strains had to be dealt with, as in quarantine stations, those strains not giving the Type I reaction would not have to be examined serologically, thus eliminating a great deal of work. In a later study, based on 558 strains which were non-agglutinable with O-group I serum, Taylor, Pandit and Read (144) found that 18 per cent fell into Heiberg's group I, again indicating that positive results in this group were of little value, although negative results excluded a very large proportion, if not all, of the strains having no serological relationship to the choleric vibrios.

An interesting extension of the work on fermentation is represented by the studies of Read (117) and Seal (126) on new differential media for vibrio isolation. It has long been a problem in cholera to demonstrate the vibrio in all clinically characteristic cases of the disease, and it has often occurred that no vibrios were obtainable in a large proportion of undoubted cholera cases. For example, Pasricha (51) found that of 502 patients suffering from clinical cholera, in the Campbell Hospital, Calcutta, no vibrios could be isolated in 62 per cent even when the most painstaking and continued efforts were made and a variety of methods and media employed. In 1936, when the annual cholera epidemic in Calcutta was more severe than usual, the same worker found that out of 1380 patients with clinical cholera only 61 per cent yielded vibrios of which 82 per cent were agglutinable with the diagnostic serum. The first thought in these instances must be that the technical methods are deficient, and accordingly steps were taken to improve the media. It was shown by Taylor, Pandit and Read (144) that the fermentation of mannose appeared to be a characteristic of the serologically authentic cholera vibrios. Goyle, in unpublished work quoted by Read (117), found that "the agglutinating vibrio could be isolated in almost pure form from an (artificial) inoculum containing that vibrio and a non-mannose fermenting vibrio, when mannose was added to simple peptone water and other media." Read (117)



demonstrated that under the usual conditions of isolation in liquid media the authentic agglutinable organism was rapidly overgrown, not only by coliform organisms, but by mannose-fermenting, non-agglutinable organisms. On the other hand, in the presence of non-mannose-fermenting vibrios the mannose-fermenting, agglutinable type could be isolated in pure culture even after 24 hours' incubation. After a large number of experiments, Read developed a bismuth-sulphite medium, based on one of those of Wilson and Blair (166), containing mannose as the only sugar. This medium in the laboratory was highly differential for all mannose-fermenting vibrios, while its pH of 9.2 kept down the growth of coliform organisms. As we have already pointed out, not all such fermenters are serologically of the O-group I type, and it was not certain how far the delicately growing vibrios of this kind would survive in face of competition with mannose-fermenting non-agglutinable vibrios.

The investigation of this point was undertaken by Seal (126), who compared Read's medium with the usual alkaline peptone water, the pH of which had been raised from 8.0 to 9.2. He found that a considerable increase in the number of agglutinable vibrios isolated from cases of clinical cholera occurred in the new medium in comparison with peptone water, and that isolations from water samples were also improved. However, the medium did not succeed in every case of clinical cholera in bringing out the agglutinating vibrio and this result was ascribed to the large number of non-agglutinating mannose fermenters present in these patients. In short, the medium, while a long step in the right direction, still left room for improvement in its specificity as a completely differential means of isolating O-group I vibrios from every case of cholera. Wilson and Reilly (167) confirmed the results of Read and Seal. Using a collection of old laboratory strains of vibrios and with some further modifications of the medium, they obtained profuse growth of true cholera vibrios and complete suppression of *Escherichia coli* and *Bacterium lactis aerogenes* from artificial emulsions in feces. The suppression of *Streptococcus faecalis* was less complete, but this organism appeared not to interfere with the growth of the choleri-

genic vibrios. *Proteus* organisms from cases of suspected dysentery and typhoid also grew on the medium and had to be differentiated from the vibrios by stained films. The medium did not differentiate completely between cholërigenic and cholera-like vibrios; of 25 strains of the latter, six grew well and 19 showed scanty growth or none at all; nor was it differential for El Tor vibrios.

*Metabolism and the chemical classification.* In a study of the respiration and the aerobic and anaerobic glycolysis of 67 vibrio strains, Linton, Mitra and Mullick (69) found that metabolism was most active in vibrios isolated from cases of cholera and belonging to chemical Group I. In Groups II and III the metabolism was less active and in Group IV it was sharply marked off in that aerobic glycolysis was practically negative. Strains of the rugose or "Medusa-head" type (Linton, 64) showed less active metabolism in every respect, notably in anaerobic glycolysis. In none of the other groups was there any difference in respect to this activity, but among the rugose strains the value for anaerobic glycolysis was only 10 per cent of that found in the others, indicating a profound modification in their growth habit. Group VI was almost as active metabolically as Group I, and Group V occupied an intermediate position. In general there occurred a correlation between metabolism, chemical structure and the sources of the strains. The most active vibrios were derived from cases of cholera, followed by Groups II and VI, which have the Protein I structure. The least active strains were of the rugose type. Such strains as these, which make but slight demands upon their environment, would appear to be the best adapted to survive under unfavorable conditions, and some evidence was found (Linton, Mitra and Seal, 72) that they were in fact more resistant to direct tropical sunlight than smooth vibrios were. This suggestion was later renewed by White (164) but it has never been submitted to any very thorough examination, and the place of these strains in cholera epidemiology is quite unknown. Later work by Linton, Mitra and Mullick (70) on the metabolism of 210 vibrio strains gave much the same result, and in a further series of 33 strains whose chemical changes

during variation were studied (Linton, Mitra and Seal, 75) the types of metabolism were also determined and found to coincide with the chemical groups. The chief interest in this series lay in the chemical and metabolic changes occurring during dissociation, and this paper will accordingly be referred to in more detail below. In general it may be said of the work on metabolism that while it could not stand alone as the sole means of separating the vibrios into significant groups, yet used in connection with chemical analysis it supported the chemical classification. Baars (7) grew authentic cholera vibrios and El Tor vibrios in a medium containing 2 per cent glucose, peptone, salts and chalk, and found that both kinds of organisms gave  $\text{CO}_2$ , organic acids and ethyl alcohol after 24 hours' incubation. In commenting on Baars' work, Gispén (36) stated: "By using another method, Baars obtained results the opposite of Linton's, in showing that the El Tor vibrios produced more  $\text{CO}_2$  than did the cholera vibrios. However, he used a peptone medium, not containing  $\text{NaHCO}_3$ , and read his results after 24 hours. Thus the two authors have not examined the same property and their results cannot be compared." Linton, Mitra and Mullick worked with a Barcroft manometer and with media containing 0.1 per cent glucose and recorded the results after 40 minutes.

Seal and Mitra (127), studying the oxidation-reduction potentials of 37 strains of known chemical composition, found that the curves obtained for individual organisms in each chemical group were distributed over ranges which merged into one another and made it impossible to differentiate one group from another. By taking the averages of the curves of the various groups it could be shown that the organisms containing Protein I (Groups I, II and VI) has a higher final potential at 72 hours than those containing Protein II (Groups III, IV and V), just as they had a higher metabolic rate. On the other hand, the changes in pH in the media during growth were the same for all the chemical groups.

*Hemolysins and the problem of the El Tor Strains.* Interest in the hemolytic properties of the vibrios arose with the isolation of the El Tor strains in 1905. Two points of view developed

about these strains, some bacteriologists maintaining that freshly isolated vibrios from clinically proven cases of cholera were not hemolytic, while others sought to prove that the isolation of hemolytic vibrios from cholera was possible. It was early recognized that differences in hemolytic power might occur with bloods from different kinds of animals and with growth on liquid or solid media. It was also demonstrated that variations in hemolytic power in the same strain might occur from time to time. Some at least of these difficulties were cleared up by van Loghem (90), who showed in 1913 that the apparent hemolysis of blood by *V. cholerae* was actually a hemodigestion, and differed essentially from the hemolysis produced by El Tor strains. The hemolysis was a true exo- or hemotoxin. Pollitzer (115) noted that vibrios which possessed both hemodigestive and hemolytic powers had a tendency to lose the former rather readily and that this change was sometimes permanent. The additional loss of hemolytic activity was rare and in his experience always transitory. As the result of continued work, technical advances toward uniformity and understanding of the hemolytic tests were made, but at the same time the question became unimportant for practical purposes, until the rediscovery of hemolytic vibrios in the Mecca Pilgrimage of 1930 and succeeding years, at the quarantine camp at El Tor, and especially the Celebes epidemic of 1937-38, brought the subject to the fore again. The new vibrios from El Tor resembled *V. cholerae* in all respects except that they were actively hemolytic and apparently not cholerigenic. Van Loghem (91) studied these strains and concluded that like the vibrios of the 1905 isolation they hemolyzed blood rather than digested it. Van Loghem summed up the problem which then arose in the form of two questions: Can a non-hemolytic true vibrio become hemolytic? And can a hemolytic strain cause cholera?

The answer to the first question is complicated by the fact that hemolysis itself is quite a variable property. Heiberg found, for example (45), that rabbit's blood was extremely susceptible to vibrio hemolysin, while goat's blood was resistant. Human, horse, and sheep blood occupied intermediate positions. He

accordingly confined his experiments to the use of goat's blood, and showed that in numerous strains a first hemolytic test would be positive and a second negative; a few strains acted in just the opposite way. He further found that a comparative test with single colonies picked from platings of the same strain had various hemolytic powers, ranging from active to negative. He concluded that "the power of hemolysis is hardly related to simple exogenous or hereditary factors, but that it seems to depend upon laws which only more complicated investigations may be able to reveal"; and that accordingly no satisfactory classification based solely on hemotoxin production was possible. The answer to the first question would thus appear to be a possible affirmative, although with many qualifications.

The second question has been brought into prominence by the Celebes epidemic (de Moor, 102, 103) and appears to have been answered in the affirmative. Cases of clinical cholera occurred in South Celebes in September and October 1937, and again in the first three months of 1938. Like cases of true cholera they showed great variation in their mortality and symptomatology. There were 18 cases with 11 deaths in 1937 and 21 cases with 19 deaths in 1938, and all of the latter were examined bacteriologically. The agent of disease appeared to extend by contact, a method of extension which has been stated by Taylor (141) to be a characteristic only of the non-hemolytic vibrios of O-group I, but this opinion appears to require modification in view of de Moor's findings. The Celebes vibrios were serologically O-group I and biochemically Heiberg's Group I, but at the same time they were strongly hemolytic and hence de Moor (102) did not consider them true cholera vibrios, although he stated that they were the cause of the highly fatal epidemic from which they were isolated. De Moor concluded that Asiatic cholera should be considered a disease in the same sense as bacillary dysentery, in which the same or a very similar disease may be due to bacteriologically different organisms. His epidemiological observations led him to conclude that the disease had not been imported into Celebes but was indigenous and endemic there.

Van Loghem (92) took a somewhat different view of these strains. He stated: "For the first time, vibrios agglutinable, hemolytic and toxic—that is to say, El Tor vibrios—have been isolated from patients suffering from an acute enteritis." He did not agree, however, that the disease was truly cholera, since it was due to a different kind of vibrio, and he accordingly proposed to call it "enteritis choleraformis El Tor," or more simply "enteritis—El Tor." It remains to be seen whether de Moor's or van Loghem's conception of this new organism and disease will prevail. Read (51) has recently reported a case of cholera in Bengal from which a hemolytic O-group I vibrio was isolated; it appears likely that at times a vibrio of this type may be choleraogenic.

The question of hemolysis in cholera vibrios, El Tor and Celebes strains was further studied by Otten (108). He pointed out the three factors which must be considered in the study of hemolysis and the interpretation of the results, viz., the age of the culture at the time it is tested for hemolysins, the method of incubation of the mixture and the way in which the blood is subjected to the hemolytic action. When compared with authentic cholera vibrios and El Tor vibrios in respect to these three factors, Otten showed that the Celebes strains occupied an intermediate position in hemolytic power. He also observed, in confirmation of Doorenbos (30), that when conditions were suitably adjusted *V. cholerae* itself constantly produced hemolysin after short periods of growth. Otten left it an open question whether the Celebes vibrios were El Tor vibrios with slightly hemolytic powers, or strongly hemolytic cholera vibrios, and confined himself to drawing attention to the fact that early hemolysis cannot be used to differentiate various types of vibrios derived from cases of cholera.

The fact that the conditions for hemolysin production need such delicate adjustment lessens the value of several observations made on large collections of strains when little or nothing is revealed about the technique which was followed. For example, Genevray and Bruneau (35), using more than 500 freshly isolated strains, found that most of them showed some hemolysin pro-

duction at 24 hours, while Kabeshima (54) found that 91 per cent of his 206 strains were hemolytic. On the other hand, de Vogel (151) reported that none of his 400 freshly isolated strains was hemolytic.

The study of the Celebes strains was taken up by Mertens and Beeuwkes (96). When very thick suspensions were incubated for one or two days, the supernatant fluid of the El Tor and Celebes strains practically always showed hemolytic properties, while only a few authentic cholera vibrios did so. Thus a strict differentiation could not be made in this way, but further work revealed two better methods. Extracting the three kinds of vibrios with acetone-alcohol, they obtained a thermostable, hemolytic agent from the El Tor and Celebes strains which was present only in small amount in two of their cholera strains. Secondly, by growing the three vibrio types on a synthetic glutamic acid medium to which goat red cells had been added, a complete differentiation was found possible. All their experiments were in accord in showing the identity of hemolytic properties of the Celebes and El Tor strains and their difference in this respect from the true cholerigenic vibrios.

Several studies attempted to correlate hemolysins with other vibrio secretions. Goyle (39) concluded that the hemolysins were true exotoxins, since they were thermolabile, antigenic and filterable. An immune serum against a given hemolytic strain neutralized the hemolysins of other strains in a fairly quantitative way, indicating that they were homologous if not identical. It is of interest to note that Vassiliadis (146) reported that the injection of non-hemolytic vibrios into rabbits gave rise to a hemolysin neutralizing antibody. Goyle found that bloods from various animals varied in susceptibility to hemolysins, but not in the same order as Heiberg had reported. Gohar (37) showed that hemolysin and exotoxin ran parallel in the El Tor vibrios, and suggested that they were identical, although his evidence was not conclusive; and the lack of toxic symptoms in patients from whom the El Tor vibrios are isolated appears to make the correlation doubtful. Bernard (12) extracted an enzyme from solid and liquid media after a strain of proteolytic vibrios had

grown on them; the enzyme was regarded as the source of the hemodigestive activity of the strain but it was not hemolytic (16). Beeuwkes (11) confirmed the method, but showed that two enzymes were present, a proteolytic and a hemolytic. Bernard later found (13) that the enzyme could be separated and concentrated with ammonium sulphate. The enzyme resembled the papainases, and was active against denatured and normal protein (14) as well as against heat-killed vibrios (15). Bernard also found (17) that by modifying his technique a hemolysin could be separated from El Tor strains but not from true cholera vibrios. Among the characteristics of this substance (18) was its thermolability at 56°, its susceptibility to ether and formalin and its resistance to toluene. Like other plant and animal hemolysins it was neutralized by cholesterol. Bernard suggested that differences in hemolysin content in different strains might be explained by assuming a free hemolysin in the El Tor group and a partially neutralized hemolysin in the true cholera vibrios. In addition he showed that both types of vibrios yielded an acetone-soluble substance, thermostable and hemolytic, which lysed living vibrios. It was quite distinct from the true hemolysins.

Doorenbos (29) discovered that the addition of a few drops of sheep blood to a saline suspension of a newly isolated vibrio led to the rapid appearance of a violet color and to the flocculation of the red cells; at the same time, the hemoagglutinin was absorbed from the culture. This phenomenon of hemoagglutination appeared suddenly in some strains and lasted only a few hours. Doorenbos also treated 70 non-hemolytic strains of vibrio with bacteriophage and found that 14 of the secondary cultures became hemolytic, concluding that the phage action caused the change. This conclusion is weakened by the observation of Heiberg (45) that strains which are free from phage will also vary in the same way. Scholtens (121) confirmed Doorenbos' work and found that the strains with hemolytic properties showed spontaneous agglutination (i.e. roughening) in normal saline suspensions and were phage-resistant and non-lysogenic. Doorenbos (31) stated that the 52 El Tor strains found between



1930 and 1936 appeared to be less hemolytic with each year's isolations; he suggested that some of these strains may be weakly hemolytic and at the same time somewhat pathogenic. Gispén (36) in a summary of much previous work on the relationship of the El Tor and the choleric vibrios, included a list of 14 characters in which the two differed and concluded that the El Tor organisms occupied a position intermediate between the pathogenic and non-pathogenic strains. His own differential test in which he sought to show that choleric vibrios became non-agglutinable after being heated while the El Tor vibrios did not change, could not be confirmed by Taylor (51), and de Moor (103) found it less clear-cut than Gispén had stated. Marras (94, 95) summarized his extensive work on the El Tor strains isolated between 1931 and 1938 in stating that they were found in healthy pilgrims and in pilgrims suffering from common maladies; that they possess a non-specific O group which is identical with the non-specific O group of the choleric vibrios; that they are cholera-red and Voges-Proskauer positive while authentic cholera vibrios are cholera-red positive and Voges-Proskauer negative; that since the Celebes strains are pathogenic they therefore cannot be El Tor strains which are non-pathogenic and do not give rise to epidemics.

*Toxin and antitoxin.* The most complete work on this subject is that of Hahn and Hirsch (43, 44), which arose as mentioned above from the work of Hirsch on vibrio metabolism (48, 49). By adding glucose and NaOH continuously to the anaerobic cultures of certain strains, they were able after a few days to obtain toxic filtrates which could be concentrated and dried and against which an antibody (antitoxin) could be formed. The M.L.D. of the stable dry toxin was 1 to 2 mg. for guinea pigs. It was interesting to note that only the hemolytic strains were toxic and that the antitoxic sera were also antihemolytic. The toxin was very sensitive to acids, not dialyzable through parchment, moderately heat-stable, and very labile in solution. It was present as early as after 8 hours' incubation and reached its maximum concentration after three days. This work was confirmed by Andu and van Niekerk (3) in so far as the production

of the exotoxin was concerned, although they believed that an endotoxin was also present. Soeleiman and van Niekerk (131) returned to this work and concluded that the toxin of Hahn and Hirsch was largely an endotoxin. An antitoxin which they prepared by injecting the toxin into rabbits had little protective effect. On the other hand, Hahn (42) used the toxin of Hahn and Hirsch to produce an antitoxin in a goat and a horse. This antitoxin was used in British India in a series of 145 cases of cholera, in which the death rate averaged 25 per cent. This mortality is higher than that found in Calcutta cholera hospitals, where intravenous saline is being administered, but the figures given in Hahn's paper mean little in any event since the time in the epidemic at which the cases were treated is not given. The serum was not used in India beyond this series of cases reported by Hahn.

Kraus and Kovacs (59) treated centrifuged supernatants of 10-day old bouillon cultures of El Tor strains with formalin and after allowing these "toxoids" to stand for eight days injected them into rabbits. In contrast to the untreated centrifugates which were highly toxic, the "toxoids" proved non-toxic and antigenic as well, and animals so treated were protected against the toxin as well as against infection with living vibrios. These workers maintained that the toxins of paracholera and El Tor vibrios are qualitatively the same as those of the authentic cholera vibrios. Kovacs (58) in continuation of this work found, however, that the toxin of El Tor vibrios gave a necrotizing reaction in the skin of man, rabbit and guinea pig, in contrast to the toxin of cholerigenic vibrios. The reaction could be prevented by mixing the toxin with its antiserum before injection. Takita (137) found, as others had done, that not all strains of vibrio are toxigenic. His method of growth in aerobiosis with large exposed surfaces was in complete contrast to that of Hahn and Hirsch. After two to seven days the filtrates were lethal for mice and rabbits. Takita produced an antibody to this toxin which he considered a true antitoxin. He stated that the neutralization of his toxin by antitoxin took place according to the multiple law, but his data indicated rather that the amounts of

antitoxin required were greatly increased with small multiples of toxin, a result which is characteristic of the endotoxins. Animals could be immunized both actively and passively and the toxin appeared not to be the same as the hemotoxin of the El Tor strains. Takita was able to differentiate his antitoxin from the toxin-neutralizing antibody which occurs irregularly in certain animals and in man. This antibody was thermolabile in contrast to the antitoxin, and did not protect animals although it neutralized the toxin *in vitro*.

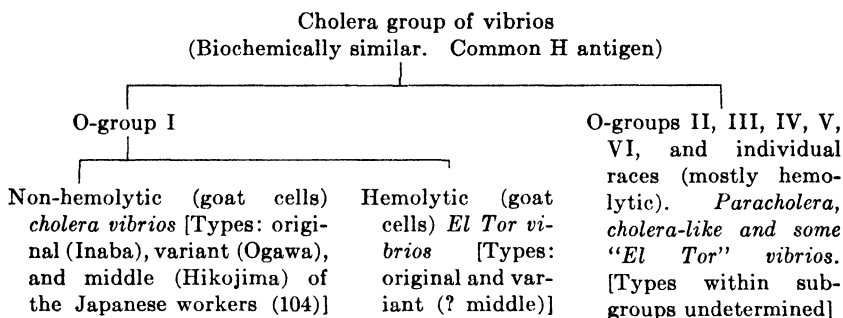
The Shwartzman phenomenon with vibrio filtrates has been studied with contradictory results. Vassiliadis (147) found that two out of three rabbits gave the reaction with a filtrate of a five-day old culture of an authentic cholera vibrio, while none of six rabbits reacted with filtrates from two El Tor strains. Linton, Harwant Singh and Seal (89), on the other hand, produced complete cross-reactions with filtrates from 20-hour cultures of the first four chemical groups, including El Tor strains, and hence concluded that the factors responsible for the reaction were not specific, as Vassiliadis had supposed.

#### THE ANTIGENIC STRUCTURE OF THE VIBRIOS

The fundamental work on the receptor structures of the cholera group was done by Balteanu (8), who showed that the vibrios, like other intestinal organisms, contained two components. In accordance with accepted terminology he named the heat-labile, flocculating portion "H" and the heat-stable granulating portion "O". Balteanu noted that the contrast between the two types of clumping was not so sharp as in other intestinal bacteria, and he attributed this difference to the monoflagellate condition of the vibrios. A suspension of flagella, prepared by shaking and centrifuging a culture, showed floccular agglutination with an antiserum against whole vibrios. The flagella were heat-labile, and when injected into rabbits yielded an antiserum which reacted with flagellar suspensions to give floccular clumps. A number of workers have filled out the picture of antigenic structure which Balteanu outlined. Aoki and Oshiro (4) studied the three types of vibrios (Inaba, Hikojima and Ogawa) originally

described by the Japanese workers (Nobechei, 104). These types arose from the same original strain and were differentiated by absorption experiments, and Aoki and Oshiro found that variation from one type to another would occur. Abdoosh (1) confirmed Balteanu's work in showing that both H and O agglutinogens were present in the vibrios, and in determining the distribution of these antigens in his collections of strains, he found that the vibrio group contained several heat-stable antigens; that all his cholericogenic vibrios had the same O antigen, and shared their H antigen with non-cholera vibrios; and that the hemolytic El Tor strains he worked with had the same H and O components as the cholericogenic vibrios. In 1931 Shousha

TABLE 3  
*Working scheme of cholera group*



recommended (129) that agglutinating sera against heated strains be used in cholera diagnosis to avoid H-group reactions.

These ideas were further extended by Gardner and Venkatraman (33), who were the first to study an adequate number of strains. They defined the cholera group as those vibrios which were biochemically and bacteriologically similar to *V. cholerae* and possessed a common "H" antigen. The scheme in table 3 taken slightly modified from their paper summarizes their findings.

Within the cholera group as thus defined they discovered a number of specific heat-stable O antigens, of which the first was the most important, as it was found only in strains known to cause cholera, and in some of the El Tor vibrios, which could be

separated, according to Gardner and Venkatraman, by the hemolytic test. We have already discussed the El Tor and Celebes vibrios and may conclude from the evidence given that they can occasionally prove pathogenic, although as these also fall into O-group I the value of the classification is only slightly lessened. Gardner and Venkatraman adopted the useful suggestion of Shousha (129) that the term "El Tor" be limited to hemolytic strains having the same specific component as the true cholera vibrios. The practical outcome of this work was to confirm the conclusion of Shousha (129) that the usual antisera for identification of cholera vibrios were useless, since their H components would agglutinate a wide range of vibrios having no connection with the disease; and Gardner and Venkatraman accordingly recommended that such sera should no longer be used in diagnostic work. This conclusion was fully confirmed by Russo (118). Otsubo (107) confirmed the serological differentiation of the Inaba and Ogawa types within O-group I.

Additional information about vibrio antigens has been given by Scholtens (119, 120), who found two serological groups in the authentic cholera vibrios. Exhaustively adsorbing the agglutinins of one cholera strain by another led to the disappearance of its agglutinating power for half of his strains and left only very slightly impaired the agglutinins for the rest. These vibrios were thus divisible into two groups: those giving rise to two kinds of agglutinins and those forming only a single agglutinin. Further work by Scholtens (122, 123) showed that the phenomenon was a general one. All strains contained agglutinogen A<sub>1</sub> and some contained B<sub>1</sub> in addition, the latter never appearing alone and probably existing in the cell as a haptene. Scholtens' work was entirely confirmed by Heiberg (46), who showed in addition that A<sub>1</sub> and B<sub>1</sub> agglutinogens made up the thermostable somatic antigen of O-group I of Gardner and Venkatraman. Strains having either A<sub>1</sub> alone or A<sub>1</sub> together with B<sub>1</sub> agglutinogens were both found to occur in cases of cholera.

Antisera prepared against dried O-group I antigens of the Inaba and Ogawa types were used in diagnostic work on a large scale in India under the auspices of the *Office International*

*d'Hygiene Publique*. The results of this work, as reported to the *Office* (34) were not published in any detail, but the conclusion was reached that these O-group I antisera specifically agglutinated the causative organisms of cholera and did not agglutinate a large group having a common H antigen, whose causal connection with cholera was doubtful. Taylor (140), reporting on the same study, felt justified in taking up the position that no series of cholera cases could be attributed to a vibrio of fixed serological type other than that of the non-hemolytic O-group I of Gardner and Venkatraman. The weight of evidence appeared to support this conclusion in general, although a number of facts have made its truth in this absolute form somewhat doubtful. In a group of 828 vibrio strains isolated from cases of cholera, Taylor (141) reported that 86.5 per cent were O-group I agglutinable, non-hemolytic and had typical biochemical characteristics, while 13.5 per cent were inagglutinable and serologically and biochemically diverse, like inagglutinable vibrios from nature (Taylor and Ahuja, 142). The vibrios which were not O-group I showed a remarkable heterogeneity, both serological and biochemical, and Gardner and Venkatraman's original five O-groups of this kind could apparently be extended almost indefinitely, besides leaving a large number of strains with individual O antigens. Taylor, Pandit and Read (144) studied 558 strains which were not O-group I agglutinable and had been isolated from clinical cases of cholera, chronic carriers and water. These were set up against 33 antisera to selected strains which were not O-group I, including O-groups II and VI of Gardner and Venkatraman. Only 311 strains (56 per cent) could be classified and these fell into 31 groups, the three largest each containing between 30 and 40 strains, and the remaining 247 strains having individual O antigens. Only 57 of the strains fell into Gardner and Venkatraman's groups II-VI. Similar results with a large collection of strains were obtained in Java by Mertens and Mochtar (97). Taylor and his colleagues believed that there was little evidence to connect any of these strains with cholera. They stated, "The heterogeneity of species actually isolated from cases, the absence of a series of cases due

to one serological type, the multiplicity of types that occur in one case, the actual identity of types in all three sources, even when no connection with cholera can be demonstrated, and the presence of these vibrios in healthy carriers without any symptoms of disease, all indicate a *chance* rather than a *causal* relationship with disease." They were careful, however, to point out two sets of observations which prevent a definite conclusion. They obtained a series of 30 strains of a type known as Rangoon Rough-1, of which 26 were from patients with cholera and on two occasions had been isolated from the same plate as the authentic cholera vibrios. They apparently hesitated to discard this group, although if it has a causal connection, which would appear to be a possibility at least, their hypothesis of the exclusive causative rôle of O-group I strains becomes untenable. A second source of doubt lay in the observation that sera prepared against strains from cases had agglutinated a higher percentage of case strains, than of carrier and water strains, while the opposite was observed with sera against carrier and water strains. If these strains had all been of the same origin, the same percentage of each should have been agglutinable with the three types of sera, and accordingly their finding pointed to a dissimilarity of origin; the authors were inclined to think that the result was an error of random sampling, although this was not proven. Taylor and Ahuja (142) later studied 90 water vibrio strains not of O-group I, from sources from which cholera contamination could be excluded, and again found that these strains were serologically diverse. A high percentage of them agglutinated to 50 per cent or over with Inaba H + O serum, indicating how confusing the use of H antiserum could be and what an advance the exclusive use of O antisera had brought about in diagnosis. These workers pointed out that this universal distribution of vibrios in water would lead to their establishment in the human intestine, and hence to their appearance in the stools of healthy individuals who subsequently acquired cholera. They did not bring any proof of this suggestion. A similar study, but in an area endemic for cholera, was made by Pasricha, Chatterjee and Das (112), who reported that none of the vibrios isolated from water, flies or

cockroaches agglutinated with O-group I serum. Lahiri and Das (60) made a similar observation on vibrios isolated from domestic animals in an endemic area, and as further evidence of the wide distribution of these vibrios, Pandit and Maitra (111) found them in 90 per cent of 105 water sources; these were not tested serologically, but biochemically they were diverse, as indeed similar vibrios were found to be in all the papers just cited.

While Gardner and Venkatraman had believed that a common H antigen was possessed by vibrios having distinct O antigens, it remained for Ahuja and Gurkirpal Singh (2) to investigate more thoroughly the H antigens of the cholera group as a whole. They worked with 219 strains not in O-group I and found that 35 per cent of them were agglutinable with H + O serum and if this serum were alone being relied upon "would have been normally diagnosed as *Vibrio cholerae*." They confirmed Vassiliadis' finding (149) of the increased sensitivity to H agglutination which results from shaking suspensions with chloroform, 53 per cent of their strains being agglutinated after this treatment and to much higher titres, as against the 35 per cent that agglutinated without the treatment. Using such chloroform-treated suspensions, they showed that some strains had H antigen identical with that of *V. cholerae*, and in others either major or minor portions were identical, while the remaining strains were individual. Finally, a high degree of flagellar heterogeneity and individuality was exhibited by strains which had no H relationship with the cholerigenic vibrios, just as Taylor and his co-workers had shown for the O antigens of the same group.

Careful perusal of the reports reviewed in this section brings out the following lines of evidence which were relied upon to show that vibrios not of O-group I did not cause cholera: first, their heterogeneity, both serological and biochemical; second, their existence in nature in places where no cholera was occurring; and third, the fact that *in vitro* they will overgrow the authentic cholera vibrios, and by analogy would do the same *in vivo*, thus accounting for the cases of clinical cholera in which the true vibrios cannot be found. While the evidence in favor of the exclusive rôle of O-group I vibrios is suggestive, it is not wholly



satisfactory, as we have already indicated. In Taylor's large series of vibrios from cholera cases, 13.5 per cent were ruled out as the causal organisms on what are, after all, wholly arbitrary serological grounds; the high degree of serological diversity which they exhibit cannot of itself be taken as proof of non-pathogenicity, and in fact the reports do show that series of these vibrios actually exist, which, if this hypothesis were not being maintained, would undoubtedly be considered as the causes of the disease in which they were found. Streptococci and dysentery bacilli are serologically diverse, but one does not on that account alone exclude some of them and include others among the pathogens. Much the same may be said for the second point: pathogenic organisms whose growth requirements are not strict are widespread even in the absence of their specific diseases, and the areas from which Taylor and his collaborators obtained these inagglutinable vibrios had been severely visited by cholera in times past. In regard to the third point, we can only state that there is no published evidence to show that the overgrowth of agglutinable by inagglutinable vibrios which occurs *in vitro* also takes place *in vivo*; this is a pure speculation. The large number of cases of cholera in which only non-agglutinable vibrios can be found is considered by Taylor to be the result of contamination of the intestine from the environment and hence the "isolation, apart from the stools of a case of cholera, of vibrios agglutinable with H serum, does not permit (us) to consider them as being *Vibrio cholerae*." This statement brings to mind the early dictum of Koch that without the isolation of a vibrio with certain arbitrarily defined characters from clinical cases, cholera could not be present. After some lives had been sacrificed to this dogma, the ban was lifted and the basis for diagnosis broadened. Taylor himself appeared to recognize this danger, for he wrote that "the typical vibrio cannot be isolated in all cases, and when a suspected case occurs, the necessary preventive measures should be instituted without awaiting such isolation." In short, cases which necessitate these measures occurred without the type of vibrio upon which Taylor and his colleagues had laid so much stress, while other vibrios did occur but because of the narrow

basis for diagnosis they are excluded as possible causal agents. It would seem wiser to find a diagnostic basis which would include these cases rather than to labor to exclude them and thus leave open the possibility of error.

Further illustrations of the difficulties which attend the attempts to incriminate a single serological type as the sole cause of cholera were given in the paper by Gardner and White (34). This included a report by Anderson on the use of O antisera in India; he found that in sporadic cases or small epidemics in Assam, strains were isolated which agglutinated with H + O sera but not with O sera of O-group I. In practically all of these cases, however, when a sufficient and often a large number of colonies had been examined, one could find at least one colony which agglutinated with O sera. According to the hypothesis it was then necessary to assume that this one strain was the cause of the condition, while the vibrios isolated in large numbers at the same time were to be considered only as casual contaminants of the bowel. In the same paper, Anderson reported a certain number of clinically undoubted cases of cholera in which the most thorough search would not reveal the "true" vibrio, although other vibrios were abundantly present. In commenting on this finding, Gardner stated that while it seemed to throw doubt upon the exclusive etiological role of O-group I vibrios, still one should consider the fact that the case was clinically typical as indicating that it had been derived from a patient with typical O-group I vibrios. Quite aside from the fact that Gardner and White presented no evidence to show that such a derivation had occurred, Anderson's findings would appear to indicate equally well that O-group I sera alone would not pick out all cholerigenic vibrios. That Anderson's findings were the result of the treatment of these cases by cholera phage was the opinion expressed by White in the same paper. While it may be true that some strains of cholerigenic vibrios after treatment *in vitro* show lessened or no agglutinability, it is purely an assumption to suppose that such changes would take place in the entirely different conditions in the intestinal tract, and no proof of this suggestion is given by White. In this connection it is interesting

to note that Taylor (143) has shown that bacteriophage treatment does not have any effect upon clinical cholera.

It would seem a fair summary of the evidence to state that while the majority of cases of cholera are probably due to a single type of organism, cases do occur in which other types are concerned, and that at times these may assume epidemic proportions, as the Celebes epidemic indicates. It is possible that if the authors who have reported on the discovery and uses of the O-group I antigen had considered the question of variation in the vibrios, a more useful basis for classification and diagnosis would have been found, but the importance of this factor has been consistently denied in their papers. In this connection the ideas of Doorenbos (32) may be briefly noted. In his view every vibrio isolated in the presence of clinical cholera may be complete or incomplete in its serological and biochemical reactions. No matter how incomplete it may be, however, it still has some potential relationship to cholera and to the complete type of vibrio. Atypical vibrios are more or less modified typical vibrios; for example, the non-agglutinable vibrio is the organism of sporadic cholera just as the agglutinable vibrio is of epidemic cholera. While much detail is given to support this view, it must still remain somewhat speculative, although it forms an interesting contrast to the rigid classification into pathogenic and non-pathogenic forms attempted by the workers already cited.

The actual distribution of the subtypes Inaba and Ogawa (table 3) has been studied by Pasricha (113), who found that 60 per cent of 438 strains from clinical cholera in Bengal were Inaba while 26 per cent were Ogawa, 3 per cent Inaba both H and O, and 11 per cent inagglutinable with all three sera. In a virulent epidemic in South India, on the other hand, Venkatraman and Pandit (150) showed that all of the 84 strains examined were of the Ogawa type. Pasricha (51) observed variation in the agglutination reaction from one type to the other, and de Moor (103) discovered in the Celebes epidemic a series of six strains from one case in which both Inaba and Ogawa O antigens as well as a common component were present, and suggested the practical usefulness of an antiserum containing both kinds of type-

specific antibodies, such as these strains would yield. It is evident that the relationship between the Inaba and Ogawa types is of the closest, as indeed one would expect from their origin.

Gardner and Venkatraman (33) had found that a few minutes' exposure of a vibrio suspension to a temperature of 100° sufficed to remove the H agglutinability, but if the organisms were to be used to prepare O antisera it was necessary to heat them in a boiling water bath for 2 hours. The chemical basis of these changes were investigated by Linton, Mitra and Seal (73). The data obtained suggested that destruction of the H antigen involved first a rapid change in the surface of the organism which was reflected in the removal of amino nitrogen and in increased surface potential, and did not progress much with long-continued heating; and second, by a progressive series of changes which involved the gradual loss of total nitrogen and amide nitrogen and a gradual disappearance of the A-fraction (p. 272); in short, a continuous mild hydrolysis of the vibrios. The first change destroyed the H antigen in the sense that the heated organisms with their heightened surface charge had lessened or no agglutinability, while the progressive changes so altered the structure that antisera prepared against it had a new specificity and range of reactivity. The rapid and slow series of changes appeared to explain Gardner and Venkatraman's findings. The amount of change which occurred during the destruction of the H antigen is shown by the following figures: 10 per cent of the total nitrogen, 12 per cent of the amino-nitrogen, between 10 and 13 per cent of the total substance of the organisms, and from 45 to 65 per cent of the polysaccharide must be removed before the organisms are in a suitable condition to yield O antisera on injection. The size of these figures appeared to discredit the view that H antigen is exclusively found in the flagellum.

Continuing this work, Linton, Mitra and Seal (74) studied the relation between surface charge (potential difference or P.D.) and agglutinability in heated and unheated suspensions. Heating for one hour at 60° greatly increased the P.D., especially at the higher salt concentrations, with a consequent lowering of agglutinability, since the P.D. then fell outside the critical zone

found by Northrop and DeKruif (105, 106). Heating for long periods at 100° did not cause much more increase in the surface charge. Vibrios of the various chemical groups could not be differentiated on the basis of their surface charges, nor could Soru (134) find any relationship between rate of migration in an electric field and the agglutination reaction. Linton, Mitra and Seal noted that the charge-reducing effect of immune serum played a dominant role, since with a high agglutinin titre flocculation would occur even when the saline was so dilute that the charge on the unsensitized organisms was of the order of  $-30.0$  millivolts. With heterologous antisera even of the same chemical group, the charge-reducing effect was much less, and might not bring the strain into the zone where agglutination would occur, even though the antiserum had combined with the vibrios to some extent, as shown by cataphoresis. Soru (132) found that vibrios had a negative sign of charge between pH 1.19 and 10.5, whereas sensitized vibrios had a similar charge between pH 4.5 and 10.5, an isoelectric zone between pH 3.6 and 4.5, and a positive charge when the acidity was greater than pH 3.6. The isoelectric range was not the same for all sera, and from Soru's data it seemed as if the higher the titre of the serum the less acid the isoelectric range. Soru concluded that agglutination was due to a modification of surface tension which came about through the adsorption of agglutinin on the cell surface. She then showed (133) that in fact such a modification in surface tension did occur, the sensitized vibrios being much lower in this respect, while normal rabbit serum and anti-typhoid serum lowered the surface tension only slightly.

#### DISSOCIATION AND ANTIGENIC STRUCTURE

Balteanu's paper (8) laid the foundation for future work not only in the serology but in the dissociation of the vibrios as well. He summarized the earlier observations which had dealt almost exclusively with questions of colony form and vibrio morphology, both of which have been more recently studied and illustrated by Seal (124). More thorough work had to await the general studies of Arkwright (5) on smooth and rough forms and of Weil and

Felix (152) on the H and O types. The first clear differentiation of smooth and rough forms in the vibrios was made by Shousha (128) in 1924, when he isolated typical representatives of each kind. He found that the rough strains were spontaneously agglutinable in normal saline and stable in more dilute saline. They were identical biochemically but otherwise exhibited the typical S and R differences.

Linton, Shrivastava and Mitra (86) found that variations in metabolism, biochemistry, serology and colony form were accompanied by changes in chemical structure as shown by an analysis of the proteins and carbohydrates. This study of the chemical basis of variation was carried out with a considerable number of strains, and was then repeated with single-cell cultures by Linton, Seal and Mitra (79). They stated that beginning with a culture descended from a single cell and having one set of characteristics and a certain chemical structure, it was possible to produce from it a new strain having another set of characteristics and a different chemical structure than its parent. The new strain accordingly fell into another chemical group than the original, and now shared the same characteristics as other strains, isolated from any source, of the same chemical structure. They emphasized that the variations which occurred always remained within the six chemical groups. In this frame-work of two proteins and three polysaccharides, the powers of synthesis and variation appeared considerable, but no other chemical constituents were found at any time. They concluded that the vibrios possessed a strictly limited capacity for variation. Linton (64) presented a report on this work to the *Office International d'Hygiene Publique*. Linton, Shrivastava and Mitra (86) also discovered another type of variation occurring in a series of strains which exhibited the well known phenomenon of variable agglutinability. These Basrah strains, so-called from their place of origin, had attracted much attention (Panayotatou, 109; Doorenbos, 29), and Linton and his co-workers correlated their variability with the fact that each strain was mixed, some of the constituent organisms having one type of polysaccharide and others another type. During growth and subculture, first

organisms containing the one type of polysaccharide would predominate and determine the agglutination reaction, and again the other type would be in the majority. Highly variable mixed strains of this kind are not common, and depending as they do upon the mechanical mixture of two kinds of vibrios, the variability which they exhibit is quite distinct from the already described change in synthesis of polysaccharides and proteins. Strains apparently of the same type as the Basrah strains were reported on by Taylor, Pandit and Read (144), who studied them serologically.

In comparing the agglutination reaction in vibrios of various chemical constitutions, Taylor and Ahuja (138) showed that antiserum against a vibrio of one chemical group would not agglutinate another member of the same chemical group in some instances. They found O-group I agglutinable strains to occur in five of the six chemical groups, the exception being the water vibrios of Group III, and drew attention to the fact that serological methods might fail to show differences in chemical structure. They also isolated from water in an area far from any case of cholera, a non-agglutinating, hemolytic vibrio which on subculturing for eight months became agglutinable with O-group I serum and non-hemolytic without at the same time undergoing any apparent variation in chemical constitution. In continuing this line of work, Taylor and Ahuja (139) found that three in-agglutinable vibrios became agglutinable with antisera against both H and O fractions of true cholera vibrios after serial passages through mice. Two of the strains exhibited changes in chemical structure at the same time, while the third remained fixed. White (161) criticized these findings and showed that the presumed mutants contained a type of phage (LL) which was absent in the parent strains. He believed that if mutation had actually occurred "it did so by profound catastrophe" but he preferred to think that the "alleged mutant cultures are not derived from the parents presented." He did not give any indication of his opinion as to their actual origin.

Also in line with the evidence that the vibrio agglutination reaction is mutable, Takano (136) produced immunological

varieties by subculturing in immune sera while at the same time biochemical, hemolytic and other characteristics were not altered. Some of his originally agglutinable strains acquired new immunological characteristics, and entirely resembled atypical strains found in cases of cholera. He also studied a strain which was originally atypical but acquired the serological characteristics of the cholera vibrio, although absorption tests showed that it still retained its original receptors. A third variation was noted in a strain which underwent no alteration in its agglutination reaction, but by absorption was found to have acquired the receptors of atypical strains. Lal, Ghosal and Mukerji (61) found that three to ten serial passages lasting from six hours to six days of vibrios of various kinds through house flies resulted in changes in chemical structure and in metabolic activity, but not in changes in fermentation reaction nor in the O-group agglutination reaction. These results, which were not due to bacteriophage contamination, recall those of Taylor and Ahuja (138) on the inability of agglutination to show differences in chemical structure. In view of the difficulties of interpretation, Lal and his co-workers did not feel justified in drawing any conclusions from their work. In attempting to carry out similar experiments on flies, Shortt (51) and Soparkar (51) could not obtain survival of vibrios in the fly longer than a few hours, and they found that extracts made from the intestine or abdomen of the fly had a vibriocidal effect. No attempt to correlate these contradictory observations on the house-fly has been published. Goyle and Sen Gupta (40) produced rough strains with the agglutinative type of growth by aging smooth strains in peptone-water cultures and also by growing them in 10 per cent immune serum in peptone-water. While their growth habit in liquid media changed, these strains did not show the characteristic rough forms of other intestinal bacteria when grown on agar. Serologically the new forms were identical with vibrios found in clinical cholera and quite distinct from smooth forms. In view of the fact that inagglutinable or less agglutinable vibrios are more common in cases toward the end of an epidemic and during convalescence than at the height of the disease, Goyle and Sen Gupta suggested



that advancing immunization was the cause and that at least some of these vibrios were identical with those which had appeared in immune serum. Their experiments lent support to the view that *V. cholerae* produced variants under natural as well as under artificial conditions.

The sum of this work is to show plainly the lability of the agglutination reaction in the vibrios, and accordingly to make one cautious about accepting complex serological classifications which have been formed upon the assumption that immunological variation will not occur.

As already described, the study of aerobic glycolysis and respiration by Linton and his collaborators had given confirmatory evidence for the existence of the six chemical groups. In a study of metabolic changes during variation, Linton, Mitra and Mullick (69) found that changes occurred in metabolism concurrent with changes in chemical structure and that in general as strains deviated further from the smooth agglutinable type, their metabolic activity lessened. Derived strains with a new chemical structure exhibited metabolic activities similar to those of the chemical group into which they now fell. More evidence for this statement was obtained in studying a large series of parent vibrios and the rough strains derived from them (Linton, Mitra and Seal, 75). These had been obtained by White (156), using his method of exposing young smooth cultures to their homologous activated antisera. Most of the organisms were lysed by this treatment, but there were some of the rough type among the survivors, and these were compared in chemical structure, metabolic activity and electrophoresis with the parent strains. The parent case strains in this series all belonged to chemical group I and it was found that the rough strains derived from them all fell into group IV, showing at the same time no change in the value for respiration, while the aerobic glycolysis, in consonance with previous findings for Group IV strains, had fallen practically to zero. The metabolic and chemical groupings were accordingly identical, and the variants resembled in these respects the typical Group IV strains found in nature. The changes of agglutinability which White recorded were not surprising in view of the fundamental nature of the chemical changes.

In continuation of studies already reviewed on physical aspects of the agglutination reaction in the vibrios, variation was found to be accompanied by changes in surface charge. In every case the change brought about by treatment with activated antisera led to an increased surface potential in the survivors (Linton, Mitra and Seal, 75). Organisms which were quite distinct in electrophoresis when in the smooth state often became similar or identical in this respect when growing in the rough state. This result may supply the physical basis for White's finding (156) that the rough vibrio strains are more generalized serologically than the smooth strains; the phenomenon of bacterial convergence, of which this supplies one instance, has long been recognized (41). In rho strains, which White derived from rough strains by the same method, the potential is higher than in the rough parent strains and much higher than in smooth strains. The rho strains were stated by White to be even more generalized serologically than the rough strains, a result which may again be related to their higher potential. The practical application of surface potentials in the agglutination reaction was brought out by Linton and Seal (78), who showed that strains might be classed as agglutinable when living and as inagglutinable or of low agglutinability when heat-killed, the difference apparently depending upon raised surface potential due to the heating. This factor had to be taken into account when diagnostic tests were being carried out.

Sulmann (135) could not isolate variants by selective subculturing or by raying the cultures with ultra-violet. Heating them at 48° for periods from 8 to 28 minutes in saline he obtained a variant which he thought had increased virulence for guinea pigs, although the differences were slight and might have been due to chance. The heating appeared to cause no other visible change in the cultures. More pronounced variation was induced by Vassiliadis (148) who shook saline suspensions of vibrios with chloroform and found that their agglutinability with O-sera was greatly reduced or had actually disappeared, while their H agglutination was now active at titres four to eight times as high as before treatment. Chloroform-extracted strains injected into rabbits gave O agglutinins but no H agglutinins, indicating that

the O antigen had not been destroyed. Vassiliadis also showed that the chloroform extraction of non-agglutinable strains led to their becoming agglutinable with anticholera sera to titres of 1:16,000. Of considerable interest was his finding that sera against inagglutinable vibrios agglutinated cholera vibrios which had been treated with chloroform; absorption tests gave similar results. The upshot of this work was to indicate the fineness of the distinction between the agglutinable and inagglutinable types of vibrios.

Yang and White (169) found that roughening in the vibrios involved loss of the specific soluble substance. The extreme rough variants were identical either when produced by treatment of smooth strains with A-type cholera phage or when colonies were picked. They believed that the change was not a modification in the fundamental architecture of the strain but that the rough forms were present in the original strain. By treating rough cultures with activated immune serum a further variant was obtained. In a subsequent communication White stated (155) that this variant had lost most of the rough receptors and would agglutinate to only about 25 per cent of titre with rough antisera. In his view this form represented "a grade of degeneracy below that of the R form," since it had lost more receptors and so departed further from the normal S type. Efforts to obtain still further degradation by exposing this so-called "rho" form to its own activated immune serum were unsuccessful. In another study (156) White prepared rough variants by cholera phage action on smooth strains or by treatment of smooth cultures with homologous activated antisera. The essential finding was that rough forms often converged and were of the same serological type irrespective of the differences which existed between the smooth parent strains. But while the rough groups were broader and more inclusive than the smooth groups, there was no general merging, and some strains were just as specific serologically in the rough as in the smooth form. The antisera against the rho forms, however, agglutinated all of his 19 rough vibrios with one exception to titre, irrespective of their source in nature, indicating that these forms possessed a still more generalized serological structure.

On the basis of chemical studies outlined above, Linton and his co-workers had put forward the view (86) that variation from smooth to rough involved a loss of polysaccharide. Their results had also indicated the existence of a second type of variation in which the actual chemical constituents changed from one structure to another. While agreeing that the first type of change did occur, White (158) denied that any positive transformation took place. He stated: "To the student of bacterial change the issue is important. On this point of fact rests the decision whether roughening is a variation by inhibition, suppression or loss, or is a positive change, a transformation. After long study of roughening in this and other groups I take my stand by the former hypothesis." The sole question at issue appeared to be whether the "characteristic" rough carbohydrate was already present but masked in the smooth forms, and was exposed as a new surface during the smooth-rough transition, or whether the organism could synthesize an entirely new polysaccharide during variation. The lines of evidence upon which White depended to prove the preexistence of a characteristic R polysaccharide in vibrios with smooth growth habit appeared to be three in number: (a) On precipitating smooth carbohydrate from solution with alcohol, small amounts of rough carbohydrate remained behind in the supernatant fluid, especially in the presence of acid, and this material reacted exclusively with rough antisera; (b) Upon exposing smooth polysaccharide to N/10 NaOH and heat for a few minutes, it no longer reacted with smooth cholera antiserum. Rough polysaccharide, similarly treated, did not change its reactivity with R antisera. The smooth polysaccharide was alkali-sensitive, the rough insensitive; (c) The rough and smooth polysaccharides were different in their cross-precipitation reactions. He concluded that "What is clear is that in roughening one of these receptors or substances, that serologically active in the S vibrio, is lost, while the other hitherto masked, intervenes in the (possibly complex) serological reactions of the variant."

In a study of these points, Linton and Mitra (68) found that technical manipulations of the polysaccharide in White's work could account for some of his results, without the necessity of

assuming the presence of two polysaccharides in the same organism. The differential precipitation of S and R polysaccharide was found to be the result of deacetylation; when working with acetylated polysaccharide some of it always became deacetylated during purification and this portion remained dissolved in acid solution in the presence of alcohol, but precipitated when the solution was made alkaline. The second point could similarly be accounted for as the result of deacetylation leading to changed serological reactivity. That R and S polysaccharide differed in their serological reactions also appeared to depend first upon the state of acetylation and second upon the transformation in type of polysaccharide during variation such as chemical analysis revealed. These results appeared to throw some doubt on the above-quoted conclusion of White.

It does not seem necessary to review the details of the controversy between Linton and White regarding the nature of variation in the vibrios, since the two views have been made clear already. References to further work may be found in papers by White (161, 163 to 165) and by Linton and his co-workers (79, 68).

Seal (125) compared cell division in rough, rugose and smooth vibrios, beginning with single cells and continuing the observations under the microscope until micro-colonies had been formed, and found that the essential difference lay in the degree to which the cells adhered to each other after division. A colony had a smooth appearance because after division the new cells slipped past each other and separated and then came to lie in a compact mass, while in the rough strains this slipping tendency was practically absent, the cells remaining in contact and forming chains, branches and bands with many open spaces and irregularities. This mode of division led in turn to the formation of angles and projections which stuck out from the colony and penetrated the agar surface. Seal ascribed the consistency of the rough colony to the binding together of the bacterial masses by these chains and irregular off-shoots which also gave the rough appearance to the growth. White (163), in a study of the finer structure of the vibrio colony, showed that either S or R forms could give rise to the rugose type of growth. Pasricha

(114), in his study of this form, had found an intracellular capsular substance and White (163) reported that the rugose condition was due to this gelatinous intercellular substance and that in some cases definite capsules were present as well. He later reported (164) the separation of the intercellular substance and showed that it was a haptene which reacted exclusively with antisera against rugose races.

The work reviewed in which single-cell cultures were found to produce strains of new chemical structure and serological reactivity make it profitable to ask whether changes in enzyme constitution may lie at the basis of these variations. Knight (55) has shown that the training of bacteria to grow on various media "is a distinct response to the chemical stimulus of the changed nutrient conditions." The changes in chemical structure were likewise a response to different types of growth media, and it seems a possibility that the adaptive enzymatic change for nutrition might carry with it in some instances the production of enzymes to synthesize new cell constituents as well. Where the cultures have descended from a single cell it is difficult to account for variation on any other basis. Linton, Seal and Mitra (79) agreed with Knight's statement that "there must be limits to the scope of adaptation which a given bacterium can undergo experimentally, since if there were not, 'specific' characters could hardly exist." The former workers pointed out that while almost nothing was known about the enzyme system of the vibrios, it might be suggested that each of the chemical types possessed the potentiality of producing all of the chemical structures of the whole group and that under various external stimuli, such as changes in the media, sometimes one and sometimes another of the constituents would be formed as an adaptive response.

#### CONCLUSION

This review shows that the study of the vibrios has proved no exception to the rule that fertile research opens up as many problems as it solves. Studies in chemical structure and serology have made considerable advances, but the correlation between

them awaits further work. The application of the concepts of somatic and flagellar antigens has defined rather than solved the problem of cholera etiology. The results obtained in the study of dissociation may prove of value when applied to epidemiology, or it may be found that the variants are merely abnormalities produced in the laboratory. These larger problems as well as lesser ones in the study of hemolytic power, metabolism and toxigenicity urgently call for study to the end that cholera may be still further reduced and confined in its Asiatic home.

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